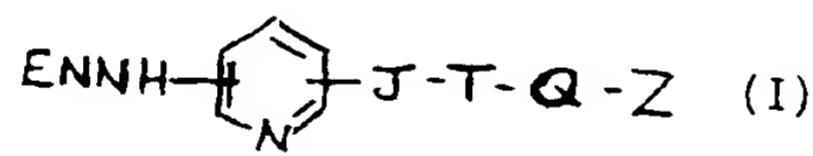


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(71) Applicant (for all designated States except US): JOHNSON MATTHEY PUBLIC LIMITED COMPANY [GB/GB]; 78 Hatton Garden, London EC1N 8JP (GB).		Published <i>With international search report.</i>	
(72) Inventors; and (75) Inventors/Applicants (for US only): ABRAMS, Michael, Jeffrey [US/US]; 999 Creek Road, Glenmore, PA 19343 (US). BRIDGER, Gary, James [GB/US]; 302 East Marshall Street, West Chester, PA 19380 (US). SCHWARTZ, David, Aaron [CA/US]; 1544 Valleda Lane, Encinitas, CA 92024 (US). PADMANABHAN, Sreenivasan [US/US]; 103 Robin Drive, Exton, PA 19341 (US). ULTEE, Michael, Evert [US/US]; 1440 Millstone River Road, Belle Mead, NJ 08502 (US).			
(54) Title: MOLECULE LABELLING USING 2-HYDRANINOPYRIDINE DERIVATIVES			
			
(57) Abstract Novel bifunctional hydrazine derivatives of general formula (I), where E is alkenyl or H ₂ , J is -CONH-, -COO-, -COS- or -NHCO-, T is alkylene or if J is -CONH-, T is the residue of an amino acid moiety, Q is a hydrophilic or cleavable moiety, and Z is an amine- and/or thiol-reactive moiety, are useful linker molecules for attaching metal ions such as ^{99m} Tc to macromolecules such as MAB's or MAB fragments.			

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Molecule labelling using 2-hydrazinopyridine derivatives

The present invention concerns improvements in molecule labelling and more especially concerns novel bifunctional hydrazine derivatives which are capable of linking metal ions, particularly technetium and rhenium, to biologically useful molecules.

Background of the Invention

Because of their high biological specificity, certain macromolecules (eg, monoclonal antibodies and fragments thereof) have been used to target 5 radioisotopes to specific in vivo sites for the purpose of imaging and/or therapy. The use of the metastable isotope of technetium, ^{99m}Tc , in diagnostic nuclear medicine is well established and the beta-emitting isotopes of rhenium ^{186}Re , ^{188}Re and ^{189}Re can be used therapeutically. A number of methods for attaching 10 technetium to macromolecules have been described. Some of these methods involve the reduction of disulphide groups in the macromolecule (usually an immunoglobulin) to thiols and the subsequent use of these groups to bind reduced Tc (eg, McKenzie et al, International Patent Publication WO 87/04164 and Bremmer et al, EP 0 271 806 A2). Direct labelling methods of this type have several potential disadvantages. The reduction of disulphide units can lead 15 to protein de-naturation and a subsequent loss in biological specificity. Also, the method cannot be used to label macromolecules lacking disulphide moieties.

Alternatively, ^{99m}Tc can be linked to macromolecules via 20 bifunctional chelates such as DTPA (D Lanteigne and D J Hnatowich, Int. J. Appl. Radiat. Isot., 35, 617, (1984)), chelating thiosemicarbazones (Y Arano et al, Int. J. Nucl. Med. Biol., 12, 425, (1985)) and diamidedithiol ligands (A Fritzberg, European Patent Appl, EP 188 256 2A). Problems associated with these methods include significant nonspecific binding of technetium (binding to

the protein at sites other than the chelating group) and slow kinetics of Tc-labelling.

5 We have previously described, in European Patent Application EP 0 384 769 A2, a novel method of modifying biological molecules (eg, monoclonal antibodies, polyclonal human IgG and ovalbumin) and smaller molecules (eg, peptides) with 2-hydrazinopyridino moieties which react with reduced Tc, eg $^{99m}\text{Tc}^{\vee}$ (glucoheptonate) to produce stable immunoreactive radioconjugates.

10

It has been demonstrated that biological molecules, (eg, monoclonal antibodies), modified to carry radioisotopes or drug molecules are metabolised in vivo and the resulting products are distributed throughout the body. In an effort to control the biodistribution of the metabolised products the chemical 15 characteristics of the modification moiety have been altered by including hydrophilic or cleavable functionalities. Paik et al (J. Nucl. Med., 30, 1693-1701 (1989) and Antibod. Immunoconjg. and Radiopharm. 3, 127-136 (1990)) have demonstrated that interposition of an ester functionality between the monoclonal antibody and the radioisotope (^{111}In) accelerated the isotope's blood clearance 20 and reduced its uptake in normal organs such as muscle, kidney, liver and spleen. This faster clearance from normal organs increased the tumour/normal organ ratios 2-3 fold. Similarly enhanced clearance in non-tumoured animals was seen by Deshpande et al (Nucl. Med. Biol., 16, 587-597 (1989)), Paik et al (Nucl. Med. Biol., 16, 475-481 (1989)), Weber et al (Bioconjugate Chem.,

1, 431-437 (1990)), and Gustavson et al (US Patent 5 112 953 (1992)) for antibodies labelled with ^{111}In or ^{99m}Tc radioisotopes via a chelator attached through an ester or disulphide function.

5 It is the object of the present invention to provide new bifunctional molecules having hydrazino groups and reactive groups spaced by hydrophilic and/or cleavable moieties which can be used to link metal ions, such as ^{99}Tc , to macromolecules so as to alter advantageously the biodistribution of the radiolabelled biological molecules.

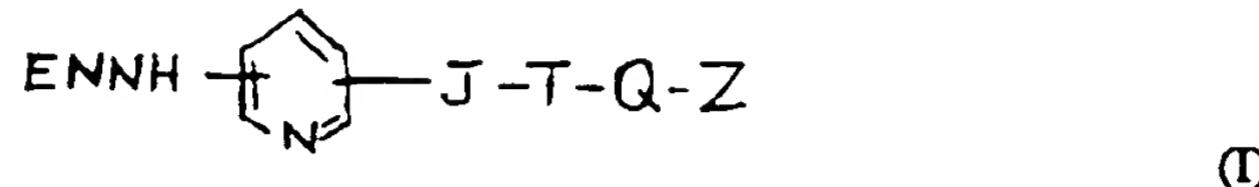
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Summary of the Invention

According to the invention, novel bifunctional hydrazine compounds containing hydrophilic (eg acid) and/or cleavable moieties (eg disulphides and esters), as well as conjugates thereof, are provided. In vivo results demonstrating tumour localisation of ^{99m}Tc labelled conjugates of compounds according to the invention with the F(ab)' fragment of monoclonal antibody C46.3 are presented below. Some of the compounds show markedly improved tumour/blood values compared to the same antibody fragment labelled via the direct method (eg Bremner et al).

The present invention provides novel compounds of general formula I,

5



in which E is an alkenyl group or represents H₂ in which case the
10 compound is in an acid addition salt form,

J is selected from the group consisting of -CO-NH-, -CO-O-,
-CO-S- and -NH-CO-,

T is an alkylene chain, or, if J is -CO-NH-, T is the residue of an
amino acid moiety,

15 Q is a hydrophilic or cleavable moiety, and

Z is an amine- and/or thiol-reactive moiety.

Where E is alkenyl, it may be straight or branched lower alkenyl,
of up to four carbon atoms.

20 Suitably, Z is thiol-reactive, such as a bromoacetate, maleimido or
disulphide or is amine-reactive, such as N-hydroxysuccinimidyl ester. The
moiety Q may be a disulphide, ester or thioester.

As specified above, when the compound of formula I is in acid addition salt form, the acid is suitably a hydrohalic acid, nitric acid, trifluoroacetic acid, tetrafluoroboric acid or sulphuric acid, but other acids may be used providing these do not interfere with the use of the compounds.

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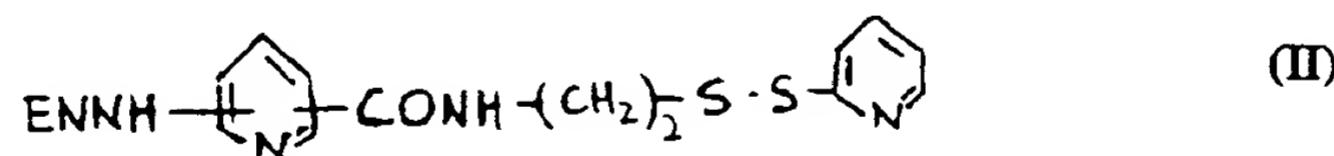
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The compounds of the invention may be synthesised by the skilled organic chemist. The molecules may be assembled in many different ways from various starting materials generally known per se. Conveniently, one of the reaction schemes set out in the accompanying drawings, schemes 1 to 4 or an obvious modification thereof is used. It will be readily appreciated that with molecules of this type, the precise starting materials and reaction conditions may be varied to give analogous processes yielding analogous products which fall within formula I. More particularly, details of the synthesis of specific compounds of formula I are given in the Examples hereinafter.

15

The invention also provides novel compounds of general formula II,

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in which E is defined above. These compounds may also find utility as linker molecules.

The invention further provides a conjugate formed by the reaction of a macromolecule with a compound according to the invention. Suitably, the macromolecule is a protein such as an immunoglobulin, for example a monoclonal antibody, or a fragment thereof. This may be achieved by a method analogous to that described by Abrams et al in J. Nucl. Med., 31, 2022 (1990).
5 Abrams et al also describe a method of radiolabelling, and in analogous manner, the conjugates of the invention may be used to produce an inventive and useful labelled macromolecule which is a metal atom, such as ^{99m}Tc or a radioisotope of Re, bound to a conjugate according to the invention.

10

As will be described in more detail hereinafter, tests on ^{99m}Tc -labelled antibody fragment conjugates show improvements over labelled fragments which do not utilise a linker molecule according to the invention or over labelled fragments utilising different linker molecules, in one or more of the characteristics of the ratio of radioactivity detected in the tumour to that in the blood, tumour to organs or clearance from the blood. Accordingly, the invention additionally provides the use of labelled macromolecules according to the invention for imaging and/or therapy, according to generally known principles.
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20

The invention will now be further described in the following Examples, which are intended to be illustrative and not limiting.

Experimental

The ^1H NMR spectra were recorded on an 300MHz Bruker AM 300 Spectrometer. All ^1H NMR spectra were recorded in DMSO-d₆ unless otherwise indicated.

Fast Atom Bombardment Mass Spectral analysis was carried out on a VG Analytical ZAB 2-SE high field mass spectrometer operating at Bacc=8kV.

10

Compound names given in brackets [] in the various examples conform to Chemical Abstracts Service Registry Index nomenclature.

15

6-Hydrazinonicotinic acid, 6-(BOC-hydrazino)nicotinic acid and succinimidyl 6-(BOC-hydrazino)nicotinate were prepared according to Abrams et al, J. Nucl. Med., 31, 2022 (1990).

20

The structures of the individual compounds prepared in the following Examples are given in the attached drawings, in which the compound numbers correspond to the Example numbers.

EXAMPLE 1**6-(BOC-Hydrazino)nicotinamido- γ -t-butyl-(L)-glutamic acid**

To a rapidly stirred solution of γ -t-butyl-(L)-glutamic acid (173g) in a mixture of dioxane (5ml) and saturated aqueous sodium bicarbonate solution (5ml) was added succinimidyl 6-(BOC-hydrazino)nicotinate (300mg, 1.0 equiv) in one portion. The mixture was stirred for 3 hours then poured into water (50ml). The pH was adjusted to pH 14 with 10 N NaOH then to pH 7 with concentrated HCl and extracted once with ethyl acetate (40ml). The pH of the aqueous phase was then lowered to pH 4 with concentrated HCl, saturated with sodium chloride and extracted with ethyl acetate (3 x 4ml). The combined organic phases were dried ($MgSO_4$) and evaporated under reduced pressure giving the product as a white powder (360mg, 96%).

15 6-(BOC-Hydrazino)nicotinamido- γ -t-butyl-(L)-glutamic acid N-hydroxy-succinimidyl ester

To a stirred solution of 6-(BOC-hydrazino)nicotinamido- γ -t-butyl-(L)-glutamic acid (360g) and N-hydroxysuccinimide (96g) in THF (10ml) under argon was added DCC (170mg) at 0°C. The mixture was stirred at 0°C for 20 2 hours then at room temperature overnight during which time a white solid had precipitated. The solid was filtered off and the filtrate evaporated under reduced pressure giving the product as a white foam (quant).

Synthesis of 1**6-Hydrazino-nicotinamido-(L)-glutamic acid-N-hydroxy succinimyl ester hydrochloride**

To a stirred solution of 6-(BOC-hydrazino)nicotinamido- γ -t-butyl-(L)-glutamic acid-N-hydroxysuccinimyl ester (150mg) in dioxane (2ml) was added a saturated solution of hydrogen chloride in dioxane (2ml). After one hour a precipitate had formed from the homogeneous solution, stirring was continued for a further 3 hours, then the solid was filtered off, washed with ether and dried giving the product as a white/yellow crystalline solid. 1 H NMR (DMSO-d₆) δ 2.10-2.29 (m, 2H), 2.39 (t, 2H, J=6.9 Hz), 2.81 (s, 4H), 4.88 (m, 1H), 6.93 (d, 1H, J=8.8 Hz), 8.18 (d, 1H, J=8.8 Hz), 8.63 (m, 2H), 9.04 (d, 1H, J=7.6 Hz, D₂O exchangeable), 9.95 (br.s, 2H, D₂O exchangeable); mass spectrum (FAB); m/e (relative intensity); 380 (60,M+1), 283(100), 201(35), 185(42), 136(40).

15

EXAMPLE 2**6-(BOC-Hydrazino)nicotinamido- γ -t-butyl-(L)-glutamyl- γ -benzyl-(L)-glutamic acid t-butyl ester**

20 A solution of 6-(BOC-hydrazino)nicotinimido- γ -t-butyl-(L)-glutamic acid (200mg), γ -benzyl-(L)-glutamic acid t-butyl ester hydrochloride (151mg, 1.0 equiv) and N-hydroxysuccinimide (53mg, 1.0 equiv) in DMF (10ml) was cooled to 0°C with stirring under argon. To this solution was added triethylamine (32ml, 1.1 equiv) followed by DCC (94mg, 1.0 equiv) in one

portion and the mixture was stirred at 0°C for 3 hours then at room temperature for 4 days during which time a white solid precipitated. The solution was diluted with ethyl acetate (50ml), cooled, and the solid filtered off. The filtrate was evaporated to dryness under reduced pressure and the oily residue partitioned in ethyl acetate (50ml) and saturated aqueous sodium bicarbonate solution (30ml), the organic layer was separated, washed exhaustively with brine, dried ($MgSO_4$) and evaporated to dryness under reduced pressure giving a white foamy solid (90%).

10 **6-(BOC-Hydrazino)nicotinamido- γ -t-butyl-(L)-glutamyl-(L)-glutamic acid t-butyl ester**

To a suspension of palladium on activated carbon (Aldrich, 10%) in ethyl acetate (5ml) was added 6-(BOC-hydrazino)nicotinamido- γ -t-butyl-(L)-glutamyl- γ -benzyl-(L)-glutamic acid t-butyl ester (300mg) and the mixture was stirred rapidly under an atmosphere of hydrogen for 6 to 8 hours (until the reaction was complete by TLC). The mixture was filtered and evaporated to dryness under reduced pressure to give a white foam. This was used directly in the next step without further purification.

20 **6-(BOC-Hydrazino)nicotinamido- γ -t-butyl-(L)-glutamyl- γ -N-hydroxy-succinimidyl-(L)-glutamic acid t-butyl ester**

The product from above (100mg) was dissolved in ethyl acetate (10ml) with N-hydroxy succinimide (18.4mg, 1.0 equiv) and cooled to 0°C with stirring under argon. To this solution was added DCC (33mg, 1.0 equiv) in one

portion and the mixture was stirred overnight at room temperature during which time a white solid precipitated. The solid was filtered off and the filtrate evaporated to dryness under reduced pressure to give the product as a white foam.

5

Synthesis of 2

6-Hydrazino-nicotinamido-(L)-glutamyl- γ -N-hydroxysuccinimidyl-(L)-glutamic acid hydrochloride

To a stirred solution of 6-(BOC-hydrazino)nicotinamido- γ -t-butyl-(L)-glutamyl- γ -N-hydroxy succinimidyl-(L)-glutamic acid t-butyl ester (20mg) in dioxane (1 ml) was added a saturated solution of hydrogen chloride in dioxane (1 ml). After one hour a precipitate had formed from the homogeneous solution, stirring was continued for a further 24 hours, then the mixture evaporated to dryness under reduced pressure. The white/yellow solid which remained was washed with ether (3 x) by decantation then evaporated and dried.

^1H NMR (DMSO- d_6 /D₂O) δ 1.80-2.20 (m, 4H), 2.31 (m, 2H), 2.76 (m, 2H), 2.80 (s, 4H), 4.24 (m, 1H), 4.42 (m, 1H), 6.90 (d, 1H, J=8.8 Hz), 8.17 (dd, 1H, J=8.8, 2.4 Hz), 8.71 (s, 1H); mass spectrum (FAB); m/e relative intensity; 509 (18, M+1).

20

EXAMPLE 3**6-(2-Propenylhydrazone)nicotinamido-3-propanol**

To a solution of succinimidyl 6-(2-propenylhydrazone)nicotinate (10g, 34.4mmol) in DMF (80ml) was added a solution of 3-aminopropanol (3.0ml, 37.9mmol) in DMF (20ml), dropwise and the mixture was stirred for 16 hours at room temperature then concentrated under reduced pressure. The residue, a pale yellow oil was dissolved in ethyl acetate (50ml) and washed with the minimum amount of water, dried (Na_2SO_4) and concentrated under reduced pressure to a pale yellow solid which was recrystallised from ethyl acetate to give the desired product as a white solid (5.6g, 65%).

N-Hydroxysuccinimidyl glutaryl chloride

Benkovic, Lerner WO 88 09380; An alternative one-pot procedure follows;

To a solution of glutaric acid anhydride (4.95g) in dichloromethane (30ml) was added N-hydroxy succinimide (5.0g) in one portion and the mixture stirred for 2.5 hours at room temperature until the reaction was complete (checked by ^1H NMR). To this mixture was added dropwise via a cannula oxalyl chloride (3.0 equiv, 2 M solution in dichloromethane, Aldrich, 65ml) during which time a strong effervescence occurred. The mixture was stirred overnight then evaporated to dryness under reduced pressure. Further dichloromethane was added and the mixture was evaporated once again. This procedure was repeated several times until the residue upon evaporation began

to crystallise. The solid was washed with ether three times by decantation and dried giving the product as a white solid.

Synthesis of 3

5 To a solution of 6-(2-propenylhydrazone)nicotinamido-3-propanol (200mg, 0.8mmol) in THF (15ml) under argon at 0°C was added triethylamine (123µl, 1.1 equiv) followed by N-hydroxy-succinimidylglutaryl chloride (200mg, 0.8mmol) in one portion and the mixture was allowed to warm to room temperature with stirring overnight. Evaporation under reduced pressure gave an oil which was re-dissolved in ethyl acetate and cooled in an ice bath giving a white crystalline precipitate of triethylamine hydrochloride which was filtered off. The filtrate was concentrated and purified by column chromatography and a short column of silica gel using 5% isopropanol in ethyl acetate as eluant. The desired product was obtained as a white powdered solid (80mg, 22%). ¹H NMR (CDCl₃) δ 1.15 (t, 3H, J=7.5 Hz), 1.96 pentet, 2H, J=6.2 Hz), 2.08 (pentet, 2H, J=7.1 Hz), 2.31-2.40 (dq, 2H, J=7.5, 5.1 Hz), 2.51 (t, 2H, 7.1 Hz), 2.73 (t, 2H, J=7.1 Hz), 2.84 (s, 4H), 3.50 (q, 2H, J=6.4 Hz), 4.24 (t, 2H, J=6.1 Hz), 6.54 (br. t, 1H, D₂O exchangeable), 7.19-7.26 (m, 2H), 7.97-8.01 (dd, 1H, J=8.8, 2.4 Hz), 8.50 (dd, 1H, J=2.4, 0.7 Hz); mass spectrum (FAB); m/e (relative intensity); 462 (100, M+1), 432 (10), 233 (42).

EXAMPLE 4**3-Hydroxypropyl-[6-(2-propenylhydrazone)]nicotinate**

To a mixture of 6-(2-propenylhydrazone)nicotinic acid (3.08g, 15.5mmol) and potassium carbonate (5.4g, 39mmol) in DMF (20ml) was added 3-bromo-1-propanol (2.58g, 18.6mmol). The reaction mixture was stirred under argon at 70°C for 16 hours. The solution was cooled and concentrated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (100ml) and extracted with water (2 x 50ml). The organic phase was dried (Na₂SO₄) and evaporated under reduced pressure to give a reddish solid. Column chromatography (silica gel; methylene chloride/methanol (95/5)) was used to isolate the product as a white solid (2.3g, 60%).

Synthesis of 4

[3-pyridinecarboxylic acid, 6-propylidenehydrazino)-3-[(bromoacetyl)oxy]propyl ester]

To a stirred solution of 3-hydroxypropyl-[6-(2-propenylhydrazone)]nicotinate (150mg, 0.6mmol) and anhydrous sodium carbonate (127mg, 1.20mmol) in dry methylene chloride (20ml), under argon, was added bromoacetyl bromide (112mg, 0.60mmol) dropwise at 0-5°C. The reaction mixture was stirred at 0-5°C for 1 hour then at room temperature for 1 hour. The solid was filtered off and the filtrate after dilution with methylene chloride (50ml) was extracted with water (25ml). The organic phase was dried (Na₂SO₄) and evaporated under reduced pressure giving the crude product as a gummy

solid. Preparative TLC (silica gel plate 1000 μ m, ethyl acetate/hexanes (2/1)) was used to isolate the product as a white solid (120mg, 54%). 1 H NMR (CDCl₃) δ 1.16 (t, 3H, J=7.5 Hz), 2.15 (m, 2H, J=6.3 Hz), 2.36 (m, 2H, J=7.6 Hz), 3.84 (s, 2H), 4.34 (t, 2H, J=6.2 Hz), 4.41 (t, 2H, J=6.3 Hz), 7.31 (d, 1H, J=10.4 Hz), 7.33 (t, 1H), 8.18 (dd, 1H, J=2.2, 9.1 Hz), 8.66 (d, J=2.2 Hz); mass spectrum (FAB); m/e (relative intensity); 374 (100, M+1), 372 (100, M+1), 294 (25), 176 (52), 121 (46).

EXAMPLE 5

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S-(2-thiopyridyl)-(L)-cysteine hydrochloride

This disulphide was synthesised by the method of P C S Chong and R S Hodges, J. Biol. Chem., (1981), 256, 5064.

15

6-(BOC-Hydrazino)nicotinamido-[S-(2-thiopyridyl)]-(L)-cysteine

To a solution of S-(2-thiopyridyl)-(L)-cysteine hydrochloride (369mg, 1.37mmol) and saturated aqueous sodium bicarbonate solution (5ml) and water (3ml) was added a solution of succinimidyl 6-(BOC-hydrazino)-pyridine-5-carboxylate (500mg, 1.42mmol) in dioxane (5ml). The reaction mixture was stirred at room temperature for 2.5 hours. Water (25ml) was added to the reaction mixture and the aqueous solution was washed with ethyl acetate to remove unreacted ester. The aqueous phase was saturated with sodium chloride and acidified to pH 3.7. The aqueous solution was extracted with ethyl acetate (2 x 25ml). The combined organic extracts were dried over magnesium

20

sulphate, filtered and concentrated to give 611mg of a sticky white solid. Ether was added to the flask and the solids scraped from the sides and isolated by filtration to give the desired product as a white solid (550mg, 82%).

5

Synthesis of 5

6-Hydrazino-nicotinamido-S-(2-thiopyridyl)-(L)-cysteine hydrochloride

A solution of hydrogen chloride (gas) in dry dioxane was prepared by bubbling hydrogen chloride into dry dioxane at a moderate rate for 5 minutes. To the hydrogen chloride/dioxane solution (5ml) was added 6-(BOC-hydrazino)nicotinamido-[S-(2-thiopyridyl)]-(L)-cysteine (50mg). The reaction mixture was stirred at room temperature for 2 hours and a white precipitate formed. The solvent was removed under reduced pressure and subsequently dried under high vacuum to give 35mg of a white amorphous solid. ¹H NMR δ 3.30 (m, 2H), 4.65 (m, 1H), 6.93 (d, 1H, J=8.6 Hz), 7.25 (m, 1H), 7.75 (m, 1H), 8.15 (d, 1H, J=8.6 Hz), 8.45 (d, 1H), 8.70 (s, 1H), 9.05 (d, 1H); mass spectrum (FAB); m/e; 366, 351, 257, 223, 202, 179.

10

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EXAMPLE 6

20

S-(2-Thio-5-nitropyridyl)-(L)-cysteine hydrochloride

4-Nitropyridine disulphide (1.98g, 6.34mmol) was added to DMF (10ml) and the mixture was heated to aid dissolution. The solution was cooled to room temperature and a solution of (L)-cysteine hydrochloride (0.5g, 3.17mmol) in DMF (6ml) was added. The reaction mixture became bright

yellow and was stirred at room temperature for 16 hours. A minor amount of precipitate formed which was removed by filtration. The filtrate was concentrated to give a thick yellow-brown oil which on treatment with dichloromethane yielded a yellow precipitate. The precipitate was isolated by filtration. The solids were dissolved in methanol with mild heating and filtered to remove white insoluble solids. The filtrate was treated with ether to induce precipitation. The yellow solid was isolated by filtration to give 240mg of the desired product. A further 120mg of product precipitated from the original dichloromethane filtrate; total yield 360mg (39%).

10

6-(BOC-Hydrazino)nicotinamido-S-(2-thio-5-nitropyridyl)-(L)-cysteine

S-(2-Thio-5-nitropyridyl)-(L)-cysteine hydrochloride (220mg, 0.70mmol) was dissolved in saturated aqueous sodium bicarbonate (5ml) and a solution of succinimidyl 6-(BOC-hydrazino)nicotinate (246g, 0.70mmol) in dioxane (5ml) was added and the reaction mixture was stirred at room temperature for 3 hours. Water (25ml) was added and the aqueous solution was washed with ethyl acetate to remove unreacted ester. The aqueous phase was acidified to pH 3.3 with 1 N HCl then saturated with sodium chloride. The acidic aqueous solution was extracted with ethyl acetate (2 x 35ml). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated to give 145mg of a yellow solid which was suspended in ether and isolated by filtration to give 35mg of the desired product. The filtrate was concentrated and treated with ether/hexanes to yield a further 95mg of desired product; total yield 125mg (35%).

Synthesis of 6**6-(Hydrazino-nicotinamido-S-(2-thio-5-nitropyridyl)-(L)-cysteine hydrochloride**

A solution of hydrogen chloride (gas) in dry dioxane was prepared
5 by bubbling hydrogen chloride into dry dioxane at a moderate rate for
5 minutes. To a hydrogen chloride/dioxane solution (5ml) was added 6-(BOC-
hydrazino)nicotinamido-S-(2-thio-5-nitro-pyridyl)-(L)-cysteine (50mg). The
reaction mixture was stirred at room temperature for 2 hours and a white
precipitate formed. The solvent was removed under reduced pressure and
10 subsequently dried under high vacuum to give 25mg of a white amorphous
solid. ^1H NMR δ 3.30 (m, 2H), 4.66 (m, 1H), 6.92 (d, 1H, $J=9.0$ Hz), 8.01
(d, 1H, $J=9.0$ Hz), 8.12 (d, 1H, $J=8.9$ Hz), 8.48 (dd, 1H, $J=8.9, 2.7$ Hz), 8.64
(s, 1H), 9.03 (d, 1H, $J=8.3$ Hz), 9.2 (br. s, 1H); mass spectrum (FAB); m/e; 411,
391, 363, 335, 307, 293, 277, 257, 201, 185, 171, 157.

15

EXAMPLE 7Synthesis of 7

Prepared from (L)-penicillamine using similar experimental
20 procedures to those described in Examples 5 and 6. ^1H NMR (DMSO- d_6) δ
1.41 (s, 3H), 1.43 (s, 3H), 4.75 (m, 1H), 6.92 (d, 1H, $J=9.1$ Hz), 7.20 (m, 1H),
7.79 (m, 2H), 8.14 (dd, 1H, $J=9.8, 2.3$ Hz), 8.39 (d, 1H, $J=4.0$ Hz), 8.54 (s,
1H); mass spectrum (FAB); m/e (relative intensity); 394 (100, M+1).

EXAMPLE 8**6-(BOC-Hydrazino)nicotinamido-3-propanol**

To a stirred solution of succinimidyl 6-(BOC-hydrazino)-nicotinate (350mg) in DMF (4ml) cooled to 0-5°C was added a solution of 3-amino-1-propanol (90mg, 1.2 equiv) in DMF (2ml). The mixture was stirred at 0-5°C for 1 hour and then at room temperature for 16 hours. The reaction mixture was concentrated to dryness to give a white solid residue. The residue was dissolved in ethyl acetate (100ml) and extracted with water (2 x 25ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the product as a white powder (330mg, 90%).

To a stirred solution of 6-(BOC-hydrazino)nicotinamido-3-propanol (296mg, 1 equiv) and anhydrous sodium carbonate (212mg, 2 equiv) in dry methylene chloride (15ml), under argon, was added bromoacetyl bromide (200mg, 1.1 equiv) dropwise at 0-5°C. The mixture was stirred at 0-5°C for $\frac{1}{2}$ hour then at room temperature for 2 hours. The solid was filtered off and the filtrate after dilution with methylene chloride (50ml) was extracted with water (25ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the crude product as a pale yellow solid. The crude product was purified by column chromatography on silica gel using methylene chloride/methanol (90/10) as the eluent, giving the pure bromoacetylated product as a white solid (267mg, 62%) was obtained.

Synthesis of 8

[Acetic acid, bromo-, 3-[[6-hydrazino-3-pyridinyl]carbonyl]amino]propoxy ester monohydrobromine)]

A solution of hydrogen bromide in ethyl acetate was prepared by 5 passing anhydrous hydrogen bromide (gas) through ethyl acetate (10ml) at a moderate rate for 5 minutes.

The above bromoacetate (90mg) was dissolved in ethyl acetate (1 ml) and HBr/ethyl acetate (2ml) was added and the reaction mixture was 10 stirred at room temperature. After 5 minutes the solution became cloudy and a precipitate formed. Stirring was continued for 2½ hours. The cloudy mixture was filtered, washed with ether (3 x 10ml) and dried under reduced pressure to yield a white solid (65mg, 71%). ^1H NMR (DMSO-d₆) δ 1.95 (t, 2H, J=6.2 Hz), 3.35 (t, 2H, J=6.2 Hz), 4.15 (s, 2H), 4.20 (t, 2H, J=6.2 Hz), 6.95 (d, 1H, J=8.8 Hz), 8.15 (dd, 1H, J=2.4, 8.8 Hz), 8.65 (d, 1H, J=2.4 Hz); mass spectrum (FAB); m/e (relative intensity); 333 (33, M+1), 331 (33, M+1), 136 (100). 15

EXAMPLE 920 **3-Hydroxypropyl 6-(BOC-hydrazino)nicotinate**

To a mixture of 6-(BOC-hydrazino)nicotinic acid (3.0g, 11.86mmol) and potassium carbonate (2.2g, 15.9mmol) in DMF (20ml) was added 3-bromo-1-propanol (2.0g, 14.39mmol). The reaction mixture was stirred under argon at 70°C for 16 hours. The solution was cooled and concentrated

to dryness under reduced pressure. The brown residue was dissolved in ethyl acetate (100ml) and extracted with water (2 x 50ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give a pale yellow oil. Column chromatography (silica gel, ethyl acetate/hexane (3/1)) was used to isolate the product as a white solid (2.28g, 60%).

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To a stirred solution of the above ester-alcohol (935mg, 3.0mmol) and anhydrous sodium carbonate (636mg, 6.0mmol) in dry methylene chloride (25ml), under argon, was added bromoacetyl bromide (290 μ l, 3.6mmol) dropwise at 0-5°C. The reaction mixture was stirred at 0-5°C for 30 minutes then at room temperature for 1 hour. The solid was filtered off and the filtrate after dilution with methylene chloride (50ml) was extracted with water (25ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the crude product as a brownish yellow sticky solid. Column chromatography (silica gel; ethyl acetate/hexane (2/1)) was used to isolate the pure product as a white solid (0.36g, 28%).

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Synthesis of 9

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A solution of hydrogen bromide in dioxane was prepared by passing anhydrous hydrogen bromide (gas) into dioxane (10ml) at a moderate rate for 5 minutes. The above bromoacetate (75mg) was dissolved in dioxane (2ml) and HBr/dioxane (2ml) was added and the reaction mixture was stirred at room temperature for 2 minutes. The cloudy mixture was filtered, washed with ether (3 x 10ml) and dried under reduced pressure to yield a white solid

(40mg, 56%). ^1H NMR (D_2O) δ 2.18 (t, 2H, $J=6.0$ Hz), 4.01 (s, 2H), 4.39 (t, 2H, $J=6.0$ Hz), 4.44 (t, 2H, $J=6.0$ Hz), 6.89 (d, 1H, $J=9.0$ Hz), 8.13 (dd, 1H, $J=2.0, 9.2$ Hz), 8.62 (d, 1H, $J=2.0$ Hz); mass spectrum (FAB); m/e (relative intensity); 334 (100, M+1), 332 (100, M+1), 212 (76), 194 (38), 154 (48), 136 (45).

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EXAMPLE 10

3-Hydroxypropyl[(6-BOC-hydrazino)nicotinamido]- γ -t-butyl-(L)-glutamate

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To a stirred solution of 6-(BOC-hydrazino)nicotinamido- γ -t-butyl-(L)-glutamic acid (1.0g, 2.29mmol) and potassium carbonate (348mg, 1.1 equiv) in DMF (5ml) was added 3-bromopropanol (227 μ l, 1.1 equiv) and the mixture was heated at 55-60°C overnight. The solution was evaporated to dryness under reduced pressure and the residue partitioned between ethyl acetate and saturated aqueous sodium bicarbonate. The organic phase was separated and washed exhaustively with water, dried (Na_2SO_4) and evaporated under reduced pressure giving a foamy white solid. The desired product was purified by column chromatography on silica gel using 90% ethylacetate in hexane as eluent, giving a white solid (900mg, 78%).

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20 The compound from above (200mg, 0.40mmol) and anhydrous sodium carbonate (84mg, 2.0 equiv) were dissolved in dichloromethane (10ml) and cooled to 0°C under argon. Bromoacetyl bromide (40 μ l, 1.2 equiv) was added dropwise and the mixture was allowed to warm to room temperature

overnight. Ethyl acetate (20ml) was added and the solution washed with saturated aqueous sodium bicarbonate, dried (Na_2SO_4) and evaporated under reduced pressure. The desired product was purified by column chromatography on silica gel using 80% ethyl acetate in hexane as eluent to give a colourless oil (40mg, 16%).

5

Synthesis of 10

The bromoacetate described above (40mg, 0.06mmol) was dissolved in anhydrous dioxane and cooled in an ice bath. Hydrogen bromide (gas) was passed through the solution for approximately 1 minute (or until saturated). The solution was allowed to stand at 0°C for 3 minutes then ether was added to precipitate the product. The white solid was allowed to settle to the bottom of the flask and the supernatant hydrogen bromide solution was decanted off with a pasteur pipette. The solid was then washed by decantation with ether ten times and the remaining traces of ether removed by evaporation under reduced pressure and drying in vacuo overnight. The product was a white solid (25mg, 77%). ^1H NMR (D_2O) δ 2.07 (pentet, 2H, $J=6.2$ Hz), 2.09-2.31 (m, 2H), 2.38 (t, 2H, $J=6.9$ Hz), 4.02 (s, 2H), 4.25 (t, 2H, $J=6.1$ Hz), 4.31 (t, 2H, $J=5.9$ Hz), 4.53 (m, 1H), 6.96 (d, 1H, $J=9.3$ Hz), 8.06-8.09 (dd, 1H, $J=9.3$, 2.1 Hz), 8.41 (d, 1H, $J=2.0$ Hz); mass spectrum (FAB); m/e (relative intensity); 463 (100, M+1), 461 (100, M+1), 383 (22), 339 (10), 237 (10).

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EXAMPLE 11

2-Carboxyethyl-2-pyridyldisulphide was prepared by the procedure according to Carlesson et al, Biochem. J. (1978), 173, 723-737.

5

Synthesis of 11

To a stirred solution of 6-(2-propenylhydrazone)nicotinamido-3-propanol (190mg, 0.8mmol) and 2-carboxyethyl-2-pyridyldisulphide (163mg, 1.0 equiv) in THF (10ml) at 0°C was added dicyclohexyl-carbodiimide (157mg, 1.0 equiv) and the mixture was stirred at 0-5°C for 72 hours during which time a white solid precipitated. The white solid was filtered off and the filtrate evaporated under reduced pressure. The residue was dissolved in ethyl acetate and cooled, which gave a further precipitate of dicyclohexyl urea ("DCU"). This procedure was repeated once again until all of the urea had been removed. The desired product was purified by column chromatography on silica gel using 5% methanol in dichloromethane as eluent. Re-crystallisation in ethyl acetate/ether gave white solid (85mg, 25%). ¹H NMR (CDCl₃) δ 1.15 (t, 3H, J=7.5 Hz), 1.95 (pentet, 2H, J=6.0 Hz), 2.36 (dq, 2H, J=5.0, 7.6 Hz), 2.80 (t, 2H, J=7.0 Hz), 3.07 (t, 2H, J=7.0 Hz), 3.50 (m, 2H), 4.04 (t, 2H, J=6.0 Hz), 6.51 (broad triplet, 1H, D₂O exchangeable), 7.07-7.12 (m, 1H), 7.17-7.21 (m, 2H), 7.60-7.72 (m, 2H), 7.95-7.99 (dd, 1H, J=8.8, 2.4 Hz), 8.22 (broad s, 1H, D₂O exchangeable), 8.42-8.45 (m, 1H), 8.55 (d, 1H, J=2.4 Hz); mass spectrum (FAB); m/e relative intensity); 448 (100, M+1), 176 (45).

EXAMPLE 12**6-(BOC hydrazino)nicotinamido-2-ethanethiol**

To a stirred solution of succinimidyl 6-(BOC-hydrazino)-nicotinate (2.0g, 5.7mmol) and triethylamine (0.8ml, 5.7mmol) in DMF (20ml) was added 2-aminoethanethiol hydrochloride (0.65g, 5.7). The reaction mixture was stirred at room temperature for 16 hours. The solution was concentrated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (100ml) and extracted with water (50ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the product as a white powder (1.19g, 67%).

To a stirred solution of 6-(BOC hydrazino)nicotinamido-2-ethanethiol (200mg) and anhydrous sodium carbonate (2 equiv) in dry methylene chloride (20ml), under argon, was added bromoacetyl bromide (1.2 equiv) dropwise at 0-5°C. The reaction mixture was stirred at 0-5°C for 30 minutes, then at room temperature for 1 hour. The solids were filtered off and the filtrate after dilution with methylene chloride (50ml) was extracted with water (25ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the crude product as a light brown solid. Column chromatography (silica gel; ethyl acetate/hexane (2/1)) was used to isolate the pure product as a pale yellow solid (110mg, 41%).

Synthesis of 12

[Acetic acid, bromo, 2-[[6-hydrazino-3-pyridinyl]carbonyl]amino]ethyl thioester, monohydrobromide]

A solution of hydrogen bromide in ethyl acetate was prepared by 5 passing anhydrous hydrogen bromide into ethyl acetate (10ml) at a moderate rate for 5 minutes. The above BOC-hydrazinopyridine derivative (22mg) was dissolved in ethyl acetate (1 ml) and HBr/ethyl acetate (2ml) was added and the reaction mixture was stirred at room temperature for 1 hour. The cloudy reaction mixture was filtered to give 12mg of a white solid (57%). ¹H NMR 10 DMSO-d₆) δ 3.15 (t, 2H), 3.45 (m, 2H), 4.45 (s, 2H), 6.95 (d, 1H), 8.15 (d, 1H), 8.65 (s, 1H); mass spectrum (FAB); m/e (relative intensity); 335 (42, M+1), 333 (42, M+1), 185 (100).

EXAMPLE 13

15

S-(2-Pyridyl)-S'(2-amino)ethyl disulphide hydrochloride

To a stirred solution of Aldrithiol (8.8g, 0.04mol) and glacial acetic acid (1.6ml) in methanol (40ml) was added dropwise 2-aminoethanethiol hydrochloride (2.3g, 0.02mol) in methanol (25ml). The bright yellow solution 20 was stirred at room temperature for 16 hours. The solution was concentrated to dryness under reduced pressure. The residue was stirred with methylene chloride (100ml) and filtered giving the product as a white powder (3.6g, 80%).

6-(2-Propenylhydrazone)-N-(2'-pyridylidithioethyl)nicotinamide**(Compound of Formula II)**

To a solution of succinimidyl 6-(2-propenylhydrazone)nicotinate (590mg, 2.0mmol) and triethylamine (0.6ml) in DMF (20ml) was added 5 2-pyridyl dithioaminoethane hydrochloride (444mg, 2mmol). The reaction mixture was stirred at room temperature for 16 hours. The solution was concentrated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (100ml) and extracted with water (2 x 50ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the product 10 as a white powder (650mg, 90%). ^1H NMR (DMSO-d6) δ 1.02 (t, 3H), 2.25 (q, 2H), 3.05 (t, 2H), 3.55 (m, 2H), 7.05 (d, 1H), 7.25 (m, 1H), 7.45 (t, 1H), 7.85 (m, 2H), 7.95 (d, 1H), 7.55 (m, 3H).

To a solution of the above disulphide (361mg, 1.00mmol) and 15 glacial acetic acid (20 μ l) in DMF (10ml) was added dropwise mercaptopropionic acid (106mg, 1.0mmol) in DMF (2ml). The reaction mixture was stirred at room temperature for 16 hours. The solution was concentrated to dryness under reduced pressure. The residue was triturated with methylene chloride (10ml) and filtered giving the product as a white powder (208mg, 20 58%).

Synthesis of 13

To a stirred solution of the above acid (108mg, 0.30mmol) and N-hydroxysuccinimide (35mg; 0.30mmol) in DMF (5ml) was added DCC

(63mg, 0.31mmol) at 0°C. The mixture was stirred at 4°C for 16 hours. The white solid (DCU) was filtered off and the filtrate concentrated to dryness under reduced pressure. The residue was triturated with ethyl acetate (25ml) and filtered. The filtrate was concentrated under reduced pressure giving 13 as a white powder (70mg, 51%). ^1H NMR (CDCl_3) δ 1.15 (t, 3H, $J=7.5$ Hz), 2.35 (m, 2H, $J=7.5$ Hz), 2.91 (s, 4H), 2.94 (t, 2H, $J=6.7$ Hz), 3.03 (m, 2H), 3.11 (m, 2H), 3.75 (q, 2H, $J=6.4$ Hz), 7.17 (d, 1H, $J=7.9$ Hz), 7.21 (t, 1H, $J=4.7$ Hz), 7.98 (dd, 1H, $J=2.4, 8.8$ Hz), 8.53 (d, 1H); mass spectrum (FAB); m/e (relative intensity); 454 (100, M+1), 253 (32), 225 (82), 176 (61), 121 (46).

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EXAMPLE 14

To a stirred solution of 6-(BOC-hydrazino)nicotinamido-t-butyl-(L)-glutamic acid (1g, 2.3mmol), triethylamine (0.32ml, 2.3mmol) and S-(2-pyridyl)-S'-(2-aminoethyl)disulphide (507mg, 2.3mmol) in DMF (10ml) was added DCC (472mg, 2.3mmol) at 0°C. The mixture was stirred at 4°C for 16 hours. The precipitated solid (DCU) was filtered and the solution concentrated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (100ml) and extracted with saturated sodium bicarbonate (50ml), brine (50ml) and water (50ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure giving the product as a white solid (1.28g, 92%).

15

20

To a solution of the above BOC-hydrazinopyridyl-glutamyl disulphide (606mg, 1.0mmol) and glacial acetic acid (100 μ l) in ethanol (25ml)

was added dropwise 3-mercaptopropionic acid (106mg, 1.0mmol) in ethanol (10ml). The reaction mixture was stirred at room temperature for 16 hours. The solution was concentrated to dryness under reduced pressure to a yellow solid. Column chromatography (silica gel; methylene chloride /methanol (3/2) and acetic acid (4% of the eluate)) was used to isolate the product as a white solid (302mg, 50%).

5 To a stirred solution of BOC-hydrazinopyridinedisulphide acid (170mg, 0.28mmol) and N-hydroxysuccinimide (33mg, 0.29mmol) in DMF (5ml) was added DCC (58.4mg, 0.28mmol) at 0°C. The reaction mixture was stirred at 4°C for 16 hours. The white solid (DCU) was filtered off and the filtrate concentrated to dryness under reduced pressure. The residue was triturated with ethyl acetate (25ml) and filtered. The filtrate was concentrated under reduced pressure giving the product as a white powder (75mg, 38%).

15

Synthesis of 14

A solution of hydrogen bromide in ethyl acetate was prepared by passing anhydrous hydrogen bromide (gas) into ethyl acetate (10ml) at a moderate rate for 5 minutes. 6-BOC-Hydrazinoglutamyl-disulphide (30mg) was dissolved in ethyl acetate (1 ml) and HBr/ethyl acetate (1 ml) was added and the reaction mixture was stirred at room temperature for 20 minutes. The slurry was filtered and dried to give a white solid (21mg, 78%). ¹H NMR (CD₃OD) δ 2.15 (m, 2H), 2.45 (t, 2H), 2.83 (s, 4H), 2.88 (m, 2H), 3.05 (m, 4H), 3.45 (m,

2H), 4.45 (m, 1H), 6.95 (d, 1H), 8.25 (d, 1H), 8.45 (s, 1H); mass spectrum (FAB); m/e (relative intensity); 543 (22, M+1), 322 (49), 269 (100), 207 (85).

5 To permit comparison in biological testing, the following compounds were prepared:

6-(BOC-Hydrazino)-3-(N-bromoacetyl)aminopyridine

10 To a stirred solution of 3-amino-6-(BOC hydrazino)pyridine (1.2g, 5.4mmol) and anhydrous sodium carbonate (682mg, 6.4mmol) in dry acetonitrile (25ml), under argon, was added bromoacetyl chloride (1.1g, 6.4mmol) dropwise at 0-5°C. The mixture was stirred at 0.5°C for ½ hour then at room temperature for 3 hours.

15 The reaction mixture was concentrated under reduced pressure and the residue was partitioned between water (50ml) and ethyl acetate (150ml). The organic phase was separated, dried (Na_2SO_4) and concentrated under reduced pressure to 25-30ml. The white solid (1.0g, 54.3%) which precipitated out was filtered and dried. ^1H NMR (DMSO- d_6): δ 1.39 (s, 9H); 3.99 (s, 2H); 6.45 (d, 1H, $J=8.8$ Hz); 7.65 (dd, 1H, $J=2.4, 8.8$ Hz); 8.15 (d, 1H, $J=2.4$ Hz).
20 Analysis: Calculated for $\text{C}_{12}\text{H}_{17}\text{N}_4\text{BrO}_3$: C - 41.75; H - 4.96; N - 16.23; Br - 23.15. Found: C - 41.87; H - 5.00; N - 16.27; Br - 23.27.

3-(N-Bromoacetyl)amino-6-hydrazinopyridine hydrobromide**(Compound 15)**

A solution of hydrogen bromide in dioxane was prepared by bubbling anhydrous hydrogen bromide (gas) through dioxane (10ml) at a moderate rate for 5 minutes. 6-(BOC-hydrazino)-3-(N-bromoacetyl)-aminopyridine (60mg) was dissolved in dioxane (2ml) and HBr/dioxane (2ml) was added and the reaction mixture was stirred at room temperature for 30 minutes during which time a precipitate had formed. The reaction mixture was stirred at room temperature for a total of 4 hours, then filtered, washed with ether (3 x 25ml) and dried under reduced pressure to give a white solid (50mg, 87.7%). ^1H NMR (D_2O): δ 4.08 (s, 2H); 7.02 (d, 1H, $J=8.8$ Hz); 7.85 (dd, 1H, $J=2.4, 8.8$ Hz); 8.25 (d, 1H, $J=2.4$ Hz).

Following the synthetic procedures described in European Patent Application No EP 0 384 769 A2, 3-amino-6-(BOC-hydrazino)-pyridine was used to prepare 3-maleimido-6-hydrazinopyridine hydrochloride (Compound 16).

Additional novel compounds according to the invention have been prepared:

EXAMPLE 173-(Benzyl oxyacetyl amido)-1-propanol

To a stirred solution of 3-amino-1-propanol (3.0g, 68.83mmol),
5 sodium bicarbonate, (14.5g, 172.6mmol), water (100ml), and dioxane (52ml)
was added a solution of benzyl oxyacetyl chloride (19.5g, 106.0mmol) in
dioxane (36ml), dropwise at 0.5°C for 4 hours. The mixture was extracted with
ethyl acetate (3 x 200ml) and the combined organic phases were washed with
brine, dried ($MgSO_4$) and evaporated under reduced pressure giving the product
10 as a clear oil (11.1g, 72%).

3-(Benzyl oxyacetyl amido)-1-propyl-(6-BOC-hydrazino)nicotinate

To a stirred solution of 6-(BOC-hydrazino)nicotinic acid (5.0g,
19.74mmol), DMF (25ml), 3-benzyl oxyacetyl amido)-1-propanol (4.4g,
15 19.74mmol) and 4-(dimethylamino)pyridine (2.48g, 19.74mmol) was added a
solution of DCC (4.48g, 21.71mmol) in DMF (10ml) dropwise at 0°C and the
mixture was stirred for 16 hours. The solution was diluted with ethyl acetate
(250ml), cooled and the solid filtered off. The filtrate was evaporated to
dryness under reduced pressure and the oily residue partitioned in ethyl acetate
20 (200ml) and saturated sodium bicarbonate solution (30ml). The organic layer
was separated, dried ($MgSO_4$) and evaporated under reduced pressure. The
product was purified by column chromatography (silica gel:ethyl acetate) to give
a white solid (5.1g, 56%).

3-Hydroxyacetylarnido)-1-propyl-6-(BOC-hydrazino)nicotinate

To a suspension of palladium on activated carbon (Aldrich 10%, 1.0g) in methanol (16ml) was added 3-(benzyloxyacetylarnido)-1-propyl-(6-BOC-hydrazino)nicotinate (1.6g, 3.48mmol) and ammonium formate (1.1g, 17.44mmol). The mixture was stirred rapidly under argon for 16 hours. The suspension was filtered through celite and evaporated under reduced pressure to give a white foam (1.0g, 78%).

3-Methanesulphonyloxyacetylarnido)-1-propyl-(6-BOC-hydrazino)nicotinate

10 To a stirred solution of 3-(hydroxyacetylarnido)-1-propyl-(6-BOC-hydrazino)nicotinate (1.0g, 2.17mmol) and triethylamine (0.41ml, 2.98mmol) in dichloromethane (20ml) was added a solution of methanesulphonyl chloride (0.23ml, 2.98mmol) in dichloromethane (5ml) dropwise at 0°C and the reaction mixture was then allowed to stir at 0°C for a further 2 hours then evaporated under reduced pressure. The residue was dissolved in ethyl acetate and washed with water, dried ($MgSO_4$) and evaporated under reduced pressure to give a white foam (1.2g, 98%).

3-(Bromoacetylarnido)-1-propyl-(6-BOC-hydrazino)nicotinate

20 To a stirred solution of 3-(methanesulphonyloxyacetylarnido)-1-propyl-(6-BOC-hydrazino)nicotinate (1.2g, 2.68mmol) in acetone (20ml) was added a solution of lithium bromide (1.7g, 26.9mmol) in acetone (30ml) dropwise and the reaction mixture was heated to reflux for 1.5 hours. After allowing to cool to room temperature the mixture was evaporated under reduced

pressure and the residue was dissolved in ethyl acetate (100ml) and washed with water (3 x 100ml). The organic layer was separated, dried (MgSO_4) and evaporated under reduced pressure and the product was purified by column chromatography (silica gel:ethyl acetate) to give a white foam (0.8g, 69%).

5 ^1H NMR (CDCl_3) δ 1.47 (s, 9H), 2.00 (pentet, 2H, $J=6.2\text{Hz}$), 3.43 (q, 2H, $J=6.3\text{Hz}$), 3.89 (s, 2H), 4.39 (t, 2H, $J=5.9\text{ Hz}$), 6.73 (d, 1H, $J=8.8\text{ Hz}$), 8.15 (dd, 1H, $J=2.2\text{ Hz}$, 8.7 Hz), 8.82 (d, H, $J=2.2\text{ Hz}$).

3-(Bromoacetylamido)-1-propyl-(6-hydrazino)nicotinate monohydrbromide

10 To a stirred solution of 3-(bromoacetylamido)-1-propyl-(6-BOC-hydrazino)nicotinate (50mg) and acetic acid (1ml) under argon was added hydrogen bromide (Aldrich, 30wt% solution in acetic acid, 1ml). The reaction mixture was stirred for 3 minutes and ether (20ml) was added immediately to precipitate the product. After stirring for 1 minute, the ether was decanted off.

15 The product was repeatedly washed with ether (6-10 times) and the final traces were removed by evaporating under reduced pressure to give a white solid (18mg, 37%). ^1H NMR (DMSO-d_6) δ 1.86 (pentet, 2H, $J=6.5\text{ Hz}$), 3.22 (q, 2H, $J=6.5\text{ Hz}$), 3.83 (s, 2H), 4.25 (t, 2H, $J=6.2\text{ Hz}$), 6.91 (d, 1H, $J=8.8\text{ Hz}$, 8.14 (dd, 1H, $J=2.2\text{ Hz}$, 8.8 Hz), 8.40 (br.t, 1H, D_2O exchangeable), 8.69

20 (d, 1H, $J=8.8\text{ Hz}$); mass spectrum (FAB); m/e (relative intensity); 333 (100, M+1), 331 (100, M+1), 253 (15), 194 (43), 178 (20).

EXAMPLE 18

Using the procedure described in Example 1, α -t-butyl-(L)-glutamic acid gave 6-BOC-hydrazino)nicotinamide- α -t-butyl-(L)-glutamic acid. Using the procedure in Example 10, 6-BOC-hydrazino)nicotinamide- α -t-butyl-(L)-glutamic acid was converted to the γ -bromoacetate derived glutamic acid linker molecule.

Final De-protection Procedure

To a stirred solution of the bromoacetate (100mg, 0.162mmol) in acetic acid (2ml) was added hydrogen bromide (Aldrich, 30wt% solution in acetic acid, 1.0ml). The reaction mixture was stirred for 3 minutes and diethyl ether (20ml) was added immediately to precipitate the product. The white solid was allowed to settle to the bottom of the flask and the supernatant hydrogen bromide solution was decanted off with a Pasteur pipette. This process was repeated ten times with ether and the remaining traces of ether removed by evaporation under reduced pressure and drying *in vacuo* overnight. The product was a white solid (50mg, 57%). ^1H NMR (MeOH-d₄) δ 2.00 (pentet, 2H, J=6.3 Hz), 2.09-2.33 (m, 2H), 2.52 (t, 2H, J=7.3 Hz), 3.95 (s, 2H), 4.15 (t, 2H, J=5.1 Hz), 4.22 (t, 2H, J=6.2 Hz), 4.63 (m, 1H), 6.97 (d, 1H, J=8.6 Hz), 8.21 (dd, 1H, J=2.1, 9.2 Hz), 8.47 (d, 1H, J=2.1 Hz); mass spectrum (FAB); m/e (relative intensity); 463 (100, M+1), 461 (100, M+1), 379 (10), 339 (10), 269 (15).

Radiolabelling Procedure

Compounds according to the invention were linked to the sulphhydryl groups of the F(ab)' fragment of monoclonal antibody C46.3 using standard methods (see Chemical Modification of Proteins, Means and Feeney, Holden-Day Inc 1971). The absence of free-SH groups after modification was confirmed by the assay of Grassetti and Murray (Arch. Biochem. Biophys. 119, 41-49, (1967)). The number of free hydrazines per F(ab)' fragment was determined by a hydrazone formation assay described by Abrams et al, (J. Nucl. Med. 31, 2022 (1990)).

10

The modified protein was then radiolabelled following a procedure similar to that described by Abrams et al, (J. Nucl. Med. 31, 2022 (1990)), and using for comparison a direct linking of ^{99m}Tc to the fragment using the Bremmer method and using the two hydrazino linker molecules 15 and 16.

15

Biodistribution Studies in Mice

Tumours were grown in 3-4 month old, athymic, nude mice by subcutaneous injection in the rear flank of 10⁶ LS174T colon carcinoma cells.

20

The ^{99m}Tc-labelled fragment conjugates were injected into mice via the retro-orbital sinus. Mice received 5-50 μ g of protein and 150-800 μ Ci of ^{99m}Tc in a volume of about 300ul of phosphate-buffered saline. The amount

injected per mouse was quantified both by the loss of weight and radioactivity of each syringe, and by the radioactivity taken up by the mouse.

Dissection was done at 4 and at 22 hours. Organs were weighed
5 on an analytical balance and their radioactivity determined on a gamma counter. Organ biodistribution was determined from these measurements using standard methods, with the blood volume of the mouse assumed to be 8%. Additional aliquots (15ul) of blood were taken at 0.5 and 2 hours for pharmacokinetic studies. All gamma counter values were corrected for radioactive decay by counting retained aliquots of the injected dose at the same time as the organs.
10 Final data are expressed as the mean of five (5) mice per group, (+/-) standard deviation, in the Table below.

From a review of the data presented in Tables 1 and 2, it is clear
15 that the linkers containing a cleavable ester function (compounds 8, 9, 10 and 17) gave improved tumour targeting compared to both direct labelling and to labelling via non-cleavable linkers (compounds 15 and 16). The tumour/blood ratios were significantly higher, due to a combination of retention in the tumour and fast clearance from the blood, as can be seen from the %Injected Dose/gram
20 tissue (Table 2). The disulphide-based linkers (compounds 5 and 7) gave conjugates with rapid blood clearance.

TABLE 1

Organ/Blood Ratios at Dissection Time for Tumour-Bearing Mice
Given C46.3 FAB, 99m Tc-Labelled Via Linkers vs Direct Means

	Linker 5 - Fab' MSR = 3.1		Linker 7 - Fab' MSR = 0.75		Linker 8 - Fab' MSR = 1.5		Linker 9 - Fab' MSR = 2.7	
Organ	4 hours	22 hours	4 hours	22 hours	4 hours	22 hours	4 hours	22 hours
Blood	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
Lung	0.59±0.09	1.37±0.40	0.50±0.08	0.90±0.21	1.01±0.27	1.60±0.49	0.70±0.04	1.11±0.10
Spleen	0.31±0.04	1.28±0.44	0.34±0.04	0.92±0.20	0.42±0.15	1.61±0.19	0.31±0.05	1.16±0.14
Liver	0.63±0.06	2.50±0.78	0.60±0.08	1.28±0.31	0.69±0.21	2.42±0.57	0.59±0.05	1.86±0.34
Kidney	54.6±6.3	282±58	13.5±2.3	61.4±14.5	116±23	316±63	55.9±6.7	121±13
Tumour	1.58±0.38	4.41±1.34	1.14±0.52	2.77±0.72	3.01±0.46	12.8±1.4	2.03±0.69	10.9±2.2
Muscle	0.33±0.18	0.80±0.43	0.20±0.11	0.36±0.08	0.20±0.05	0.24±0.05	0.14±0.02	0.18±0.02

	Linker 10 - Fab' MSR = 4.5		Linker 12 - Fab' MSR = 0.87		Linker 17 - Fab' MSR = 3.0	
Organ	4 hours	22 hours	4 hours	22 hours	4 hours	22 hours
Blood	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
Lung	0.75±0.33	1.47±0.11	0.59±0.07	0.90±0.25	0.77±0.13	1.82±0.45
Spleen	0.37±0.16	1.88±0.44	0.38±0.08	1.09±0.36	0.55±0.07	3.14±0.89
Liver	0.77±0.21	4.20±1.01	0.74±0.18	1.91±0.56	0.76±0.04	5.03±2.51
Kidney	54.6±4.9	254±30	23.5±5.3	128±33	107±7	598±200
Tumour	1.98±0.43	15.6±6.1	1.55±0.86	3.97±2.02	2.12±0.81	12.8±2.3
Muscle	0.16±0.03	0.35±0.05	0.38±0.18	0.68±0.18	0.73±0.39	2.42±1.15

	*Linker 15 - Fab' MSR = 0.62		*Linker 16 - Fab' MSR = 0.95		*Direct Labelled Fab'	
Organ	4 hours not done	22 hours	4 hours not done	22 hours	4 hours	22 hours
Blood		(1.00)		(1.00)	(1.00)	(1.00)
Lung		0.55±0.21		1.12±0.30	0.70±0.11	0.85±0.09
Spleen		0.42±0.08		1.54±0.38	0.37±0.05	0.71±0.10
Liver		0.65±0.06		2.71±0.56	0.73±0.05	1.53±0.28
Kidney		41.5±15.2		155±46	69.2±14.1	114±14
Tumour		2.00±0.59		5.09±1.06	2.39±0.55	4.02±1.13
Muscle		0.24±0.14		0.95±0.41	0.17±0.04	0.20±0.20

Notes:

1. All data are averages from 5 mice \pm standard deviation
2. MSR = Molar Substitution Ratio, the number of hydrazines incorporated per Fab' fragment.
3. *Data from conjugates of non-cleavable linkers and direct labelling for comparison.
4. >95% of 99m Tc was bound by Fab' as assayed by standard thin-layer chromatography techniques used in nuclear medicine.

TABLE 2

% Injected Dose Per Gram Tissue at Dissection Time for Tumour-Bearing Mice Given C46.3 Fab' 99m Tc-Labelled Via Linkers vs Direct Means

Organ	Linker 5 - Fab' MSR = 3.1		Linker 7 - Fab' MSR = 0.75		Linker 8 - Fab' MSR = 1.5		Linker 9 - Fab' MSR = 2.7	
	4 hours	22 hours	4 hours	22 hours	4 hours	22 hours	4 hours	22 hours
Blood	1.29 \pm 0.13	0.23 \pm 0.09	3.11 \pm 0.73	0.85 \pm 0.10	1.95 \pm 0.21	0.29 \pm 0.02	2.18 \pm 0.20	0.35 \pm 0.03
Lung	0.76 \pm 0.11	0.30 \pm 0.06	1.50 \pm 0.17	0.75 \pm 0.12	1.93 \pm 0.38	0.47 \pm 0.16	1.53 \pm 0.08	0.39 \pm 0.06
Spleen	0.40 \pm 0.02	0.28 \pm 0.09	1.05 \pm 0.28	0.76 \pm 0.07	0.80 \pm 0.19	0.47 \pm 0.06	0.66 \pm 0.12	0.40 \pm 0.07
Liver	0.81 \pm 0.10	0.54 \pm 0.07	1.83 \pm 0.27	1.06 \pm 0.13	1.32 \pm 0.24	0.70 \pm 0.16	1.28 \pm 0.07	0.63 \pm 0.08
Kidney	70.1 \pm 6.6	62.3 \pm 11.3	40.6 \pm 4.0	51.0 \pm 6.2	223 \pm 27	92.1 \pm 20.0	121 \pm 12	41.8 \pm 6.7
Tumour	1.99 \pm 0.71	0.96 \pm 0.16	3.43 \pm 1.57	2.30 \pm 0.48	5.87 \pm 1.03	3.72 \pm 0.58	4.45 \pm 1.71	3.74 \pm 0.65
Muscle	0.44 \pm 0.29	0.18 \pm 0.13	0.58 \pm 0.23	0.30 \pm 0.03	0.39 \pm 0.04	0.07 \pm 0.02	0.31 \pm 0.06	0.06 \pm 0.01

Organ	Linker 10 - Fab'		Linker 12 - Fab'		Linker 17 - Fab'	
	MSR = 4.5	MSR = 0.87	MSR = 3.0	MSR = 0.87	MSR = 3.0	MSR = 3.0
Blood	2.63±0.26	0.35±0.06	1.92±0.42	0.59±0.11	1.55±0.15	0.24±0.09
Lung	2.10±0.89	0.51±0.12	1.11±0.20	0.51±0.07	1.18±0.19	0.40±0.03
Spleen	1.03±0.35	0.64±0.12	0.71±0.11	0.62±0.13	0.86±0.14	0.69±0.10
Liver	2.14±0.46	1.43±0.20	1.37±0.15	1.09±0.17	1.18±0.17	1.05±0.36
Kidney	154±15	87.6±11.5	43.4±4.6	72.9±10.0	167±23	127±14
Tumour	5.38±0.83	5.18±1.60	2.83±1.41	2.19±0.62	3.29±1.23	2.88±0.57
Muscle	0.44±0.08	0.12±0.01	0.74±0.37	0.39±0.05	1.17±0.69	0.56±0.27

Organ	*Linker 15 - Fab'		*Linker 16 - Fab'		* Direct Labelled Fab'	
	MSR = 0.62	MSR = 0.95	MSR = 0.62	MSR = 0.95	MSR = 0.62	MSR = 0.95
Blood	4 hours not done	22 hours	4 hours not done	22 hours	4 hours	22 hours
Lung		2.69±0.29		0.72±0.12	1.65±0.10	0.35±0.04
Spleen		1.48±0.57		0.79±0.19	1.15±0.18	0.30±0.03
Liver		1.12±0.10		1.10±0.28	0.62±0.08	0.25±0.04
Kidney		1.72±0.17		1.91±0.31	1.21±0.10	0.53±0.10
Tumour		104±20		117±23	114±23	40.1±5.8
Muscle		5.25±1.00		3.62±0.72	3.97±1.04	1.41±0.41
	0.62±0.28			0.72±0.40	0.28±0.06	0.07±0.01

Notes:

1. All data are averages from 5 mice ± standard deviation.
2. MSR = Molar Substitution Ratio, the number of hydrazines incorporated per Fab' fragment.
3. *Data from conjugates of non-cleavable linkers and direct labelling shown for comparison.
4. >95% of ^{99m}Tc was bound by Fab' as assayed by standard thin-layer chromatography techniques used in nuclear medicine.

CLAIMS

1. A compound of general formula I,

5



10

in which E is an alkenyl group or represents H₂ in which case the compound is in acid addition salt form,

J is selected from -CO-NH-, -CO-O-, -CO-S- and -NH-CO-,

15 T is an alkylene chain, or, if J is -CO-NH-, T is the residue of an amino acid moiety,

Q is a hydrophilic or cleavable moiety, and

Z is an amine- and/or thiol-reactive moiety.

20 2. A compound according to claim 1, in which in formula I, when E is an alkenyl group, it is straight or branched chain lower alkenyl.

25 3. A compound according to claim 1 or 2, in which in formula I, -Y-Z is an active ester, α -bromoacetyl, α -bromoacetamidyl or a mixed substituted or unsubstituted pyridyl disulphide.

- #### 4. A compound of general formula Ia,



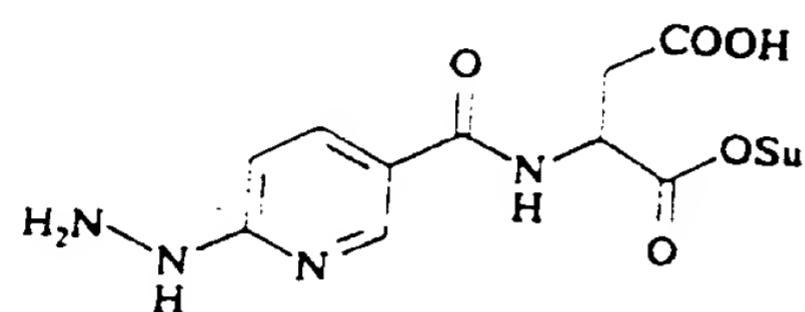
wherein E is as defined in claim 1.

R is H or CH₃, and

R is H or NO_2 .

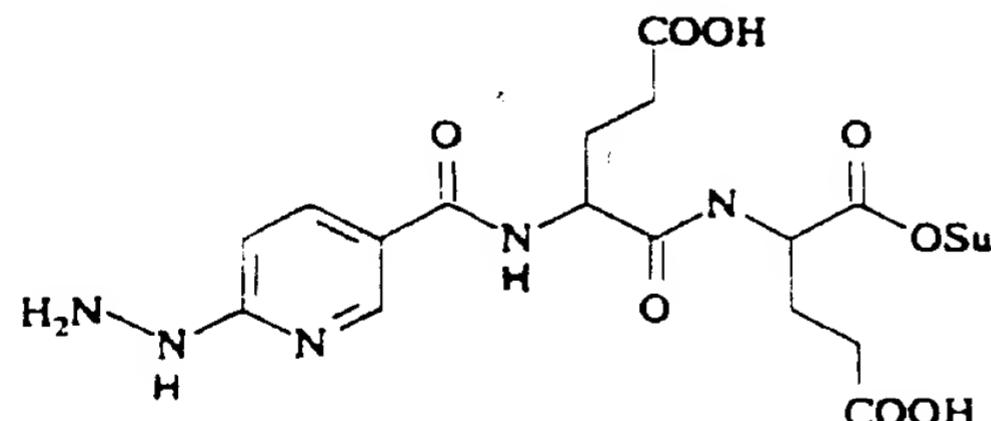
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5. The compound of claim 1 which is:



and its acid addition salts.

15 6. The compound of claim 1 which is:

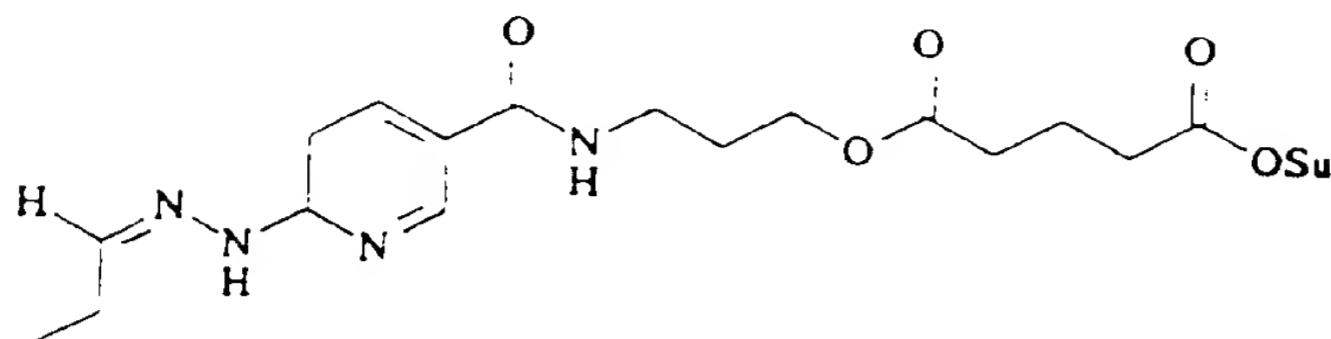


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and its acid addition salts.

7. The compound of claim 1 which is:

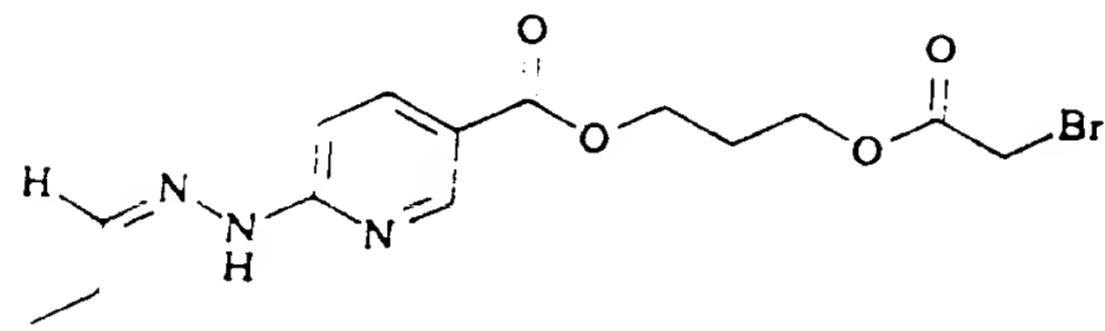
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and its acid addition salts.

8. The compound of claim 1 which is:

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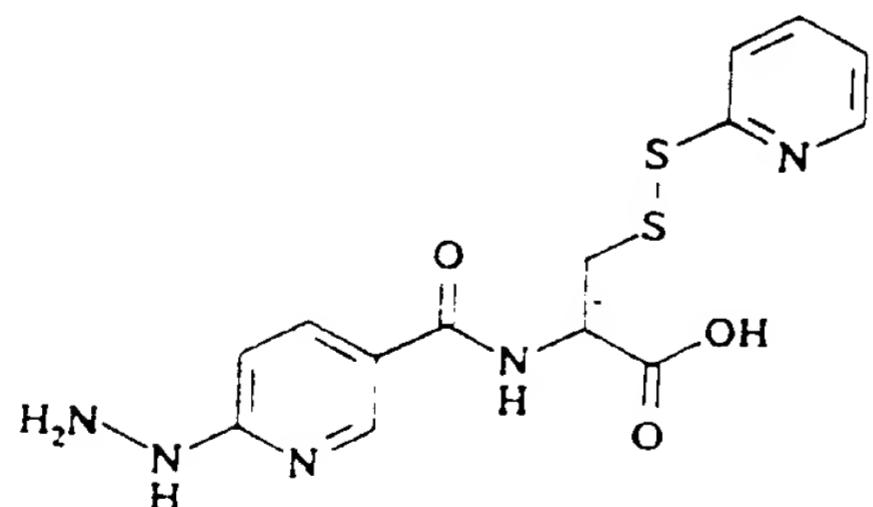


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and its acid addition salts.

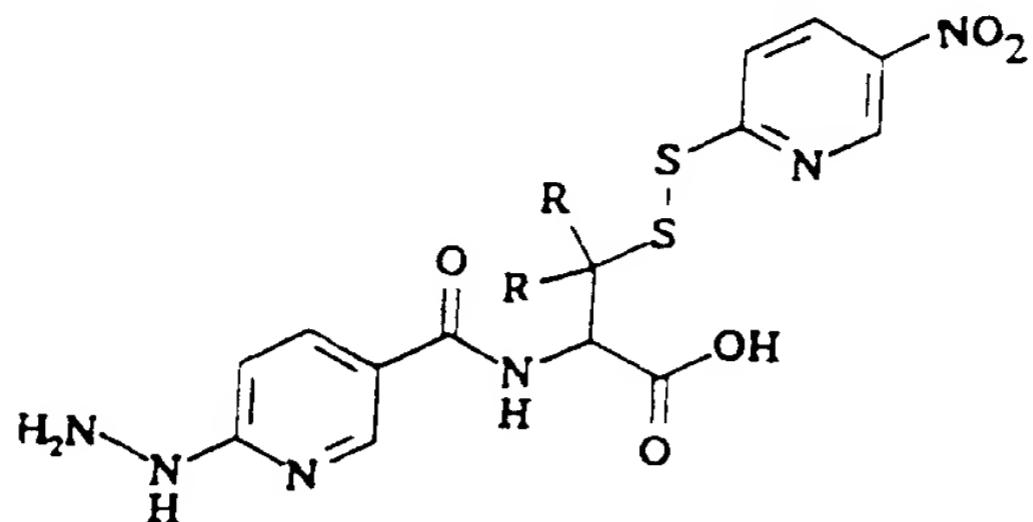
9. The compound of claim 1 which is:

20



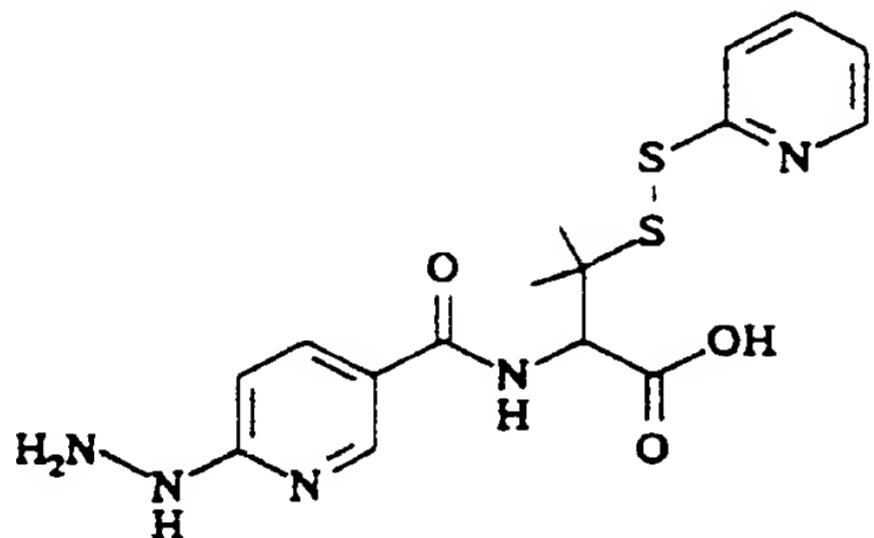
and its acid addition salts.

10. The compound of claim 1 which is:



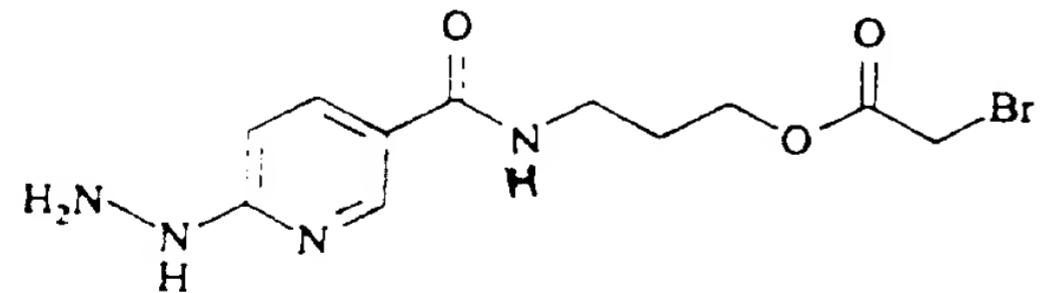
and its acid addition salts.

11. The compound of claim 1 which is:



15 and its acid addition salts.

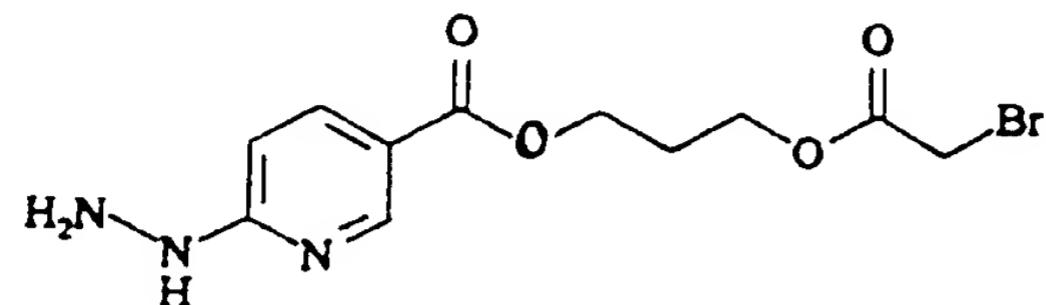
12. The compound of claim 1 which is:



and its acid addition salts.

13. The compound of claim 1 which is:

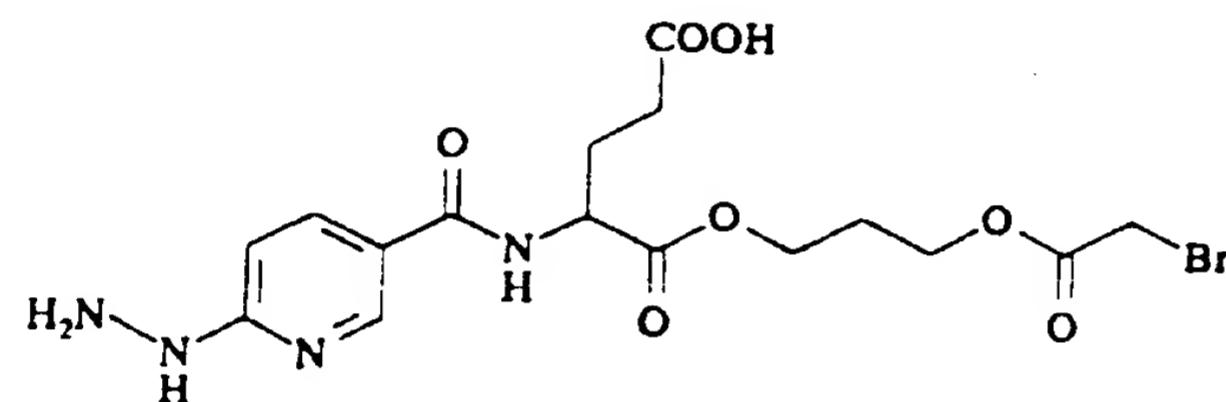
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and its acid addition salts.

14. The compound of claim 1 which is:

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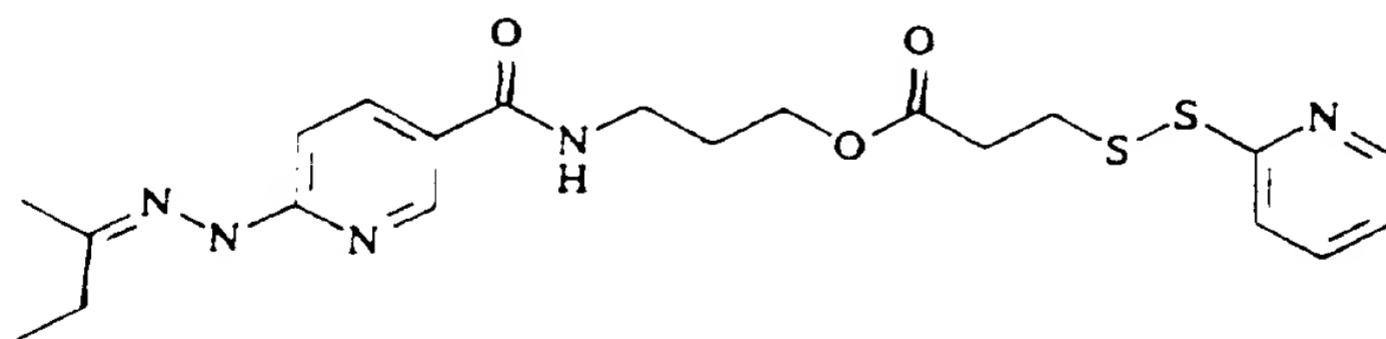


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and its acid addition salts.

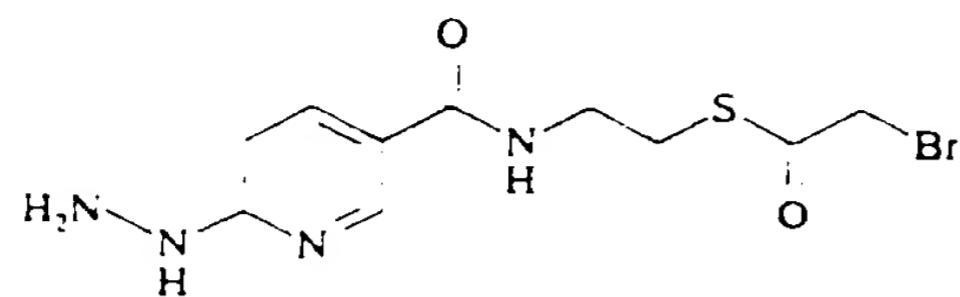
15. The compound of claim 1 which is:

20



and its acid addition salts.

16. The compound of claim 1 which is:

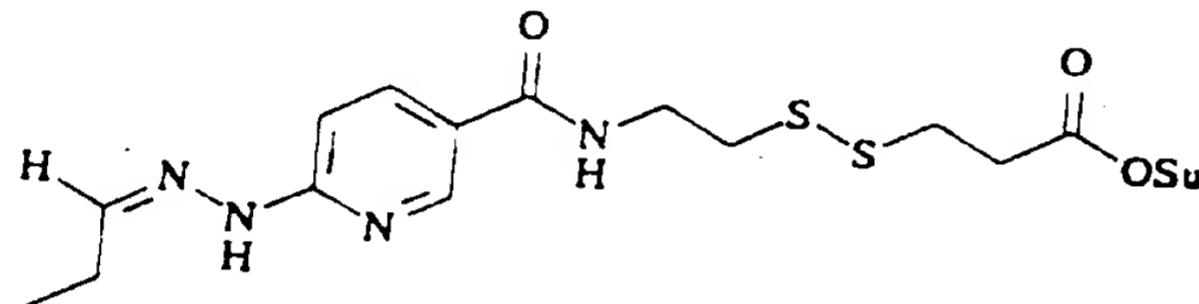


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and its acid addition salts.

17. The compound of claim 1 which is:

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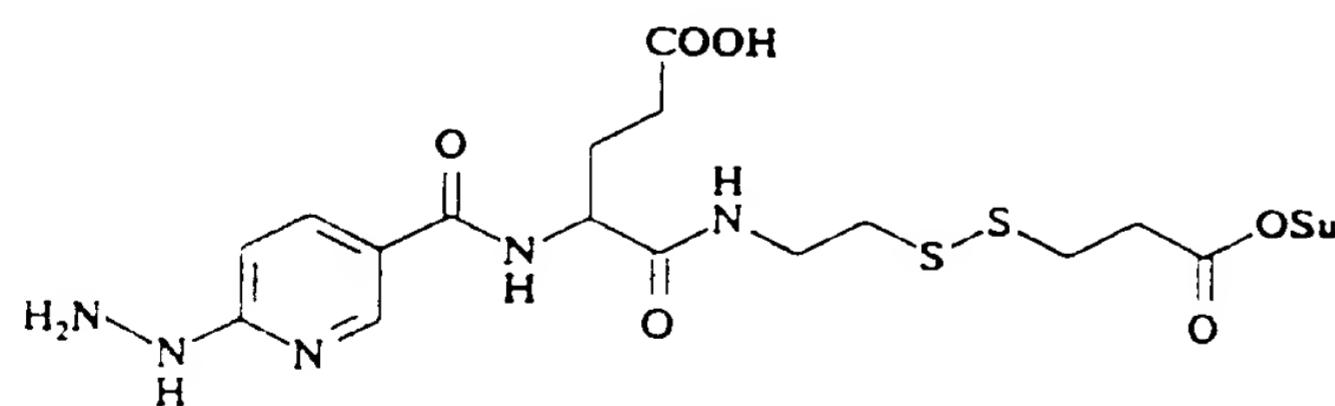


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and its acid addition salts.

18. The compound of claim 1 which is:

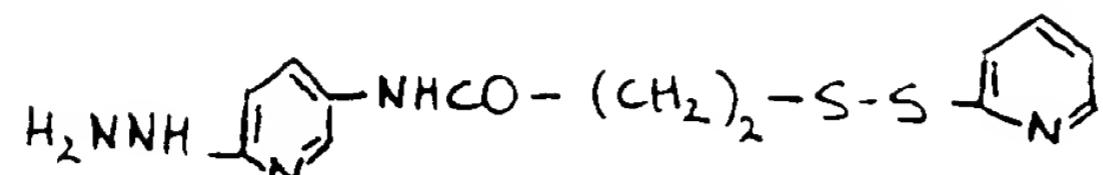
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and its acid addition salts.

- 48 -

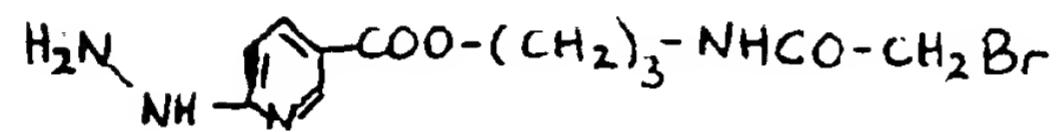
19. The compound of claim 1 which is:



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and its acid addition salts.

10 20. The compound of claim 1 which is:

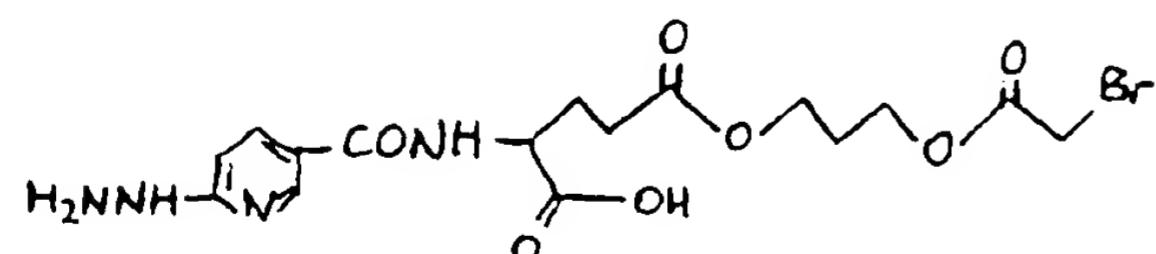


15

and its acid addition salts.

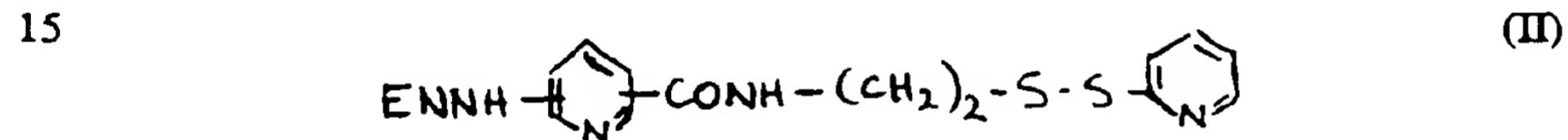
21. The compound of claim 1 which is:

20



and its acid addition salts.

22. A conjugate formed by the reaction of a macromolecule with a compound according to any one of the preceding claims.
- 5 23. A conjugate according to claim 20, wherein the macromolecule comprises an immunoglobulin or a fragment thereof.
24. A labelled macromolecule comprising a metal atom bound to a conjugate according to claim 20 or 21.
- 10 25. A labelled macromolecule according to claim 22, wherein the metal is selected from radioisotopes of Tc and Re.
26. A compound of general formula II

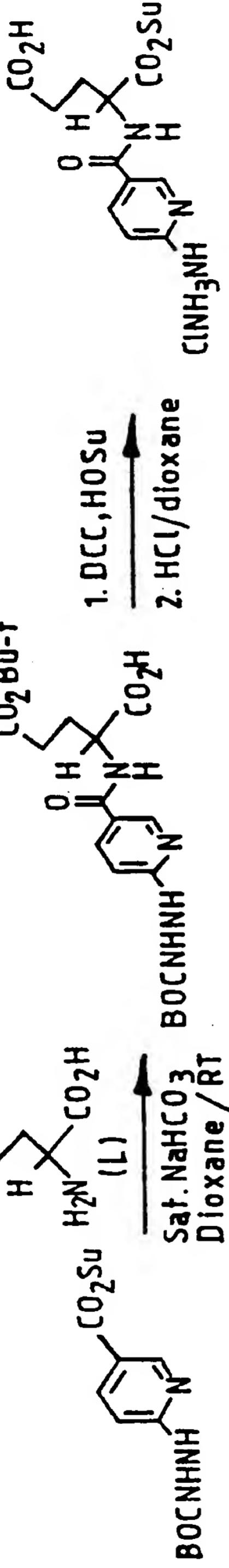


in which E is as defined in claim 1.

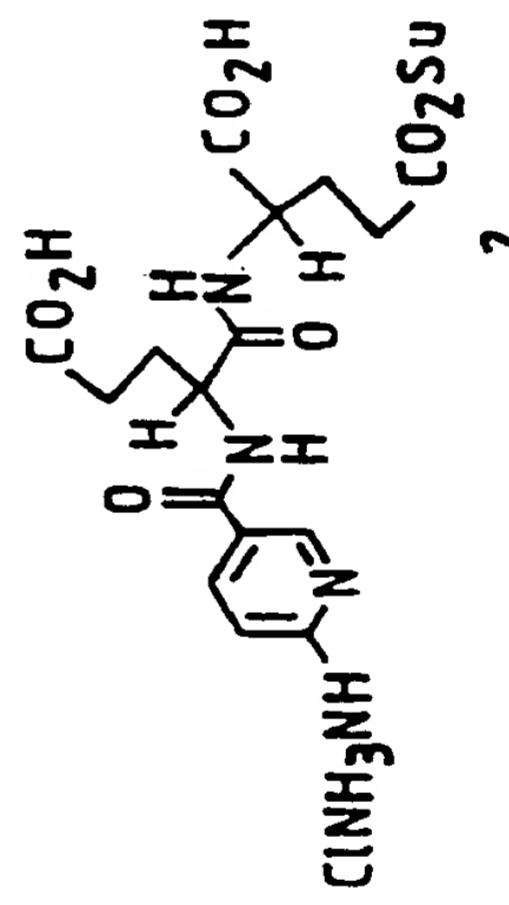
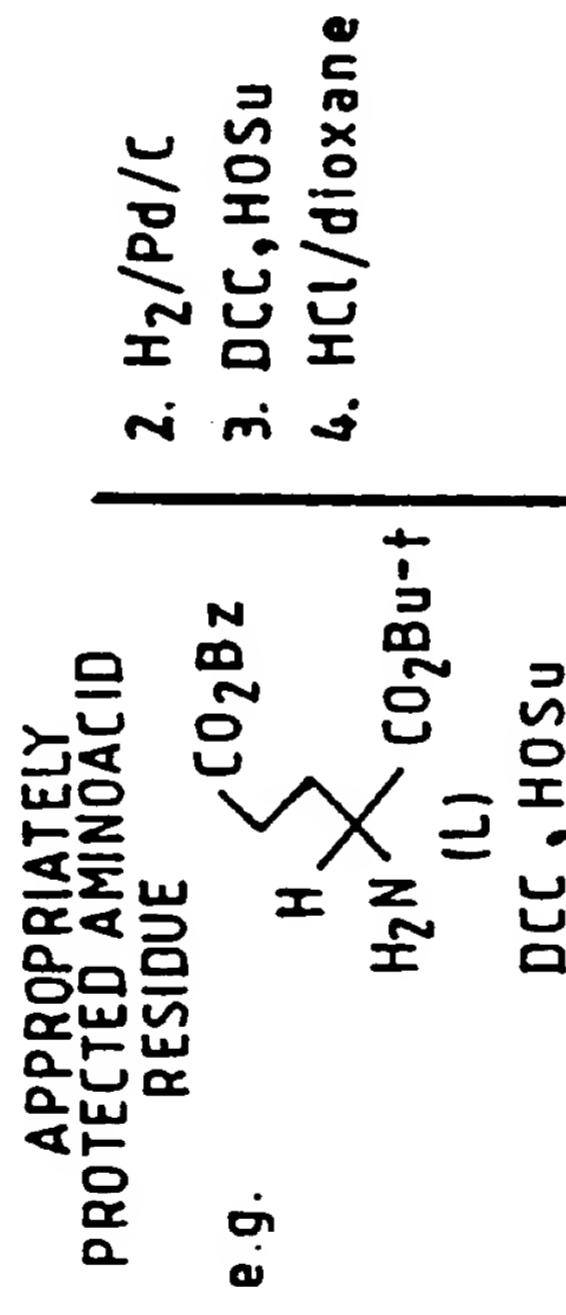
SCHEME 1

APPROPRIATELY PROTECTED AMINOACID RESIDUE

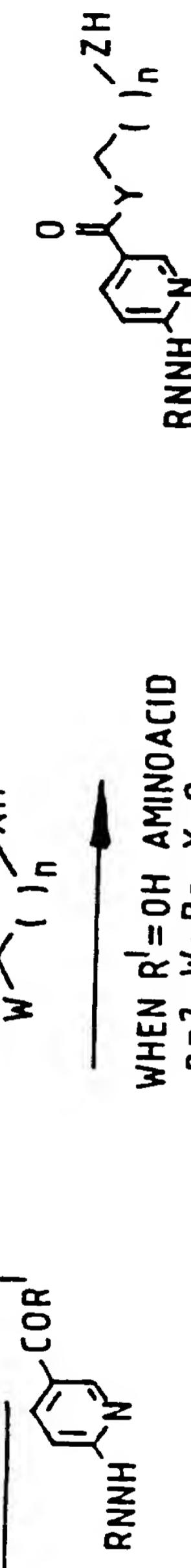
e.g.



APPROPRIATELY PROTECTED AMINOACID RESIDUE

e.g.
 

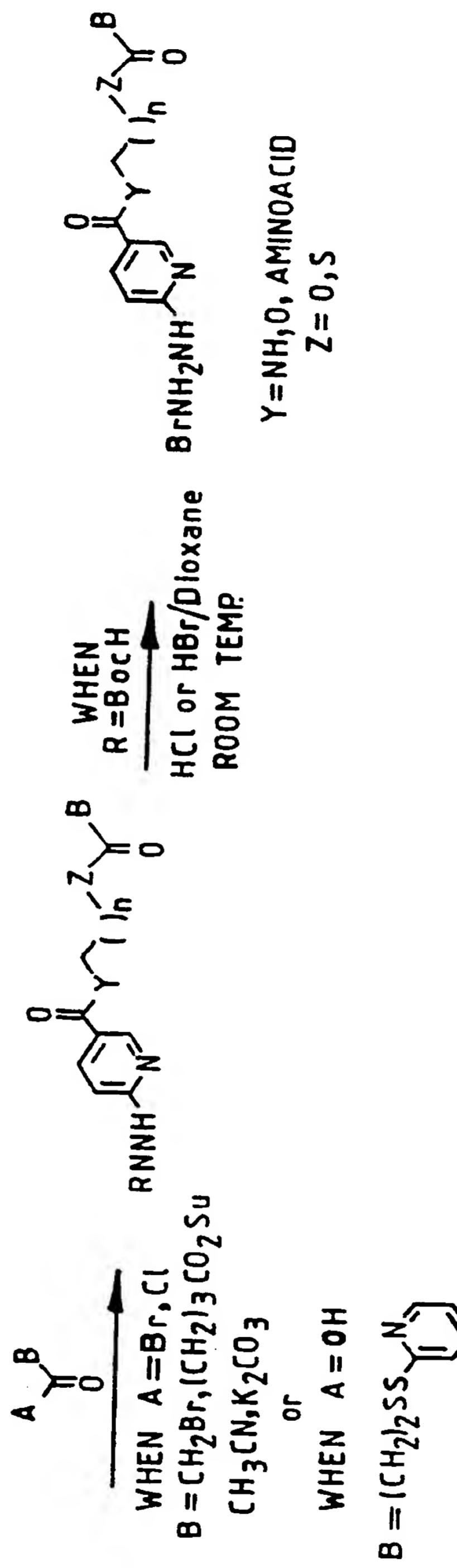
SUBSTITUTE SHEET

SCHEME 2

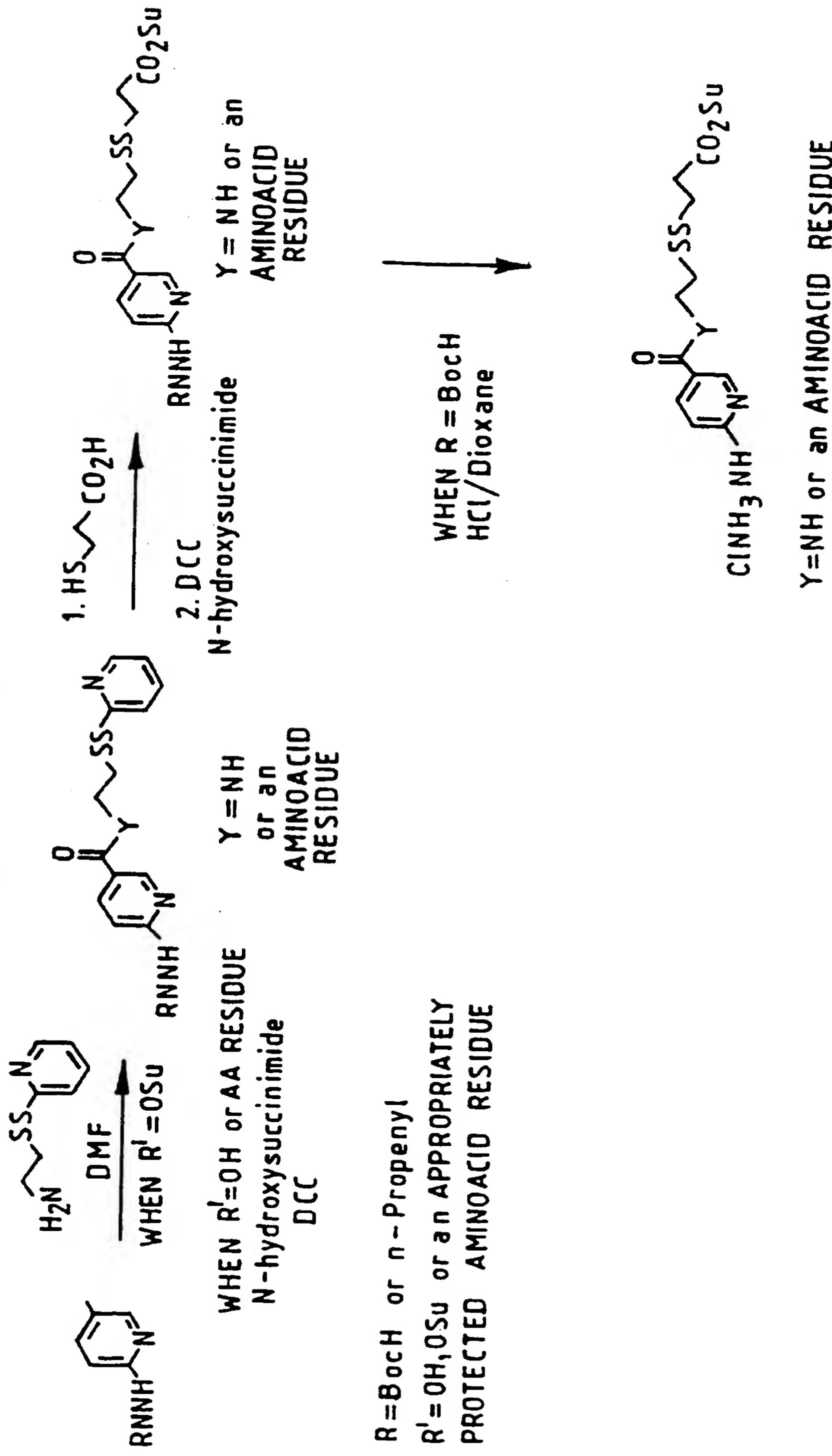
R = BocH, n - Propenyl
 R' OH, OSu or APPROPRIATELY
 PROTECTED AMINO ACID RESIDUE

WHEN R' = OH AMINOACID
 n = 2, W = Br, X = O
 $\text{K}_2\text{CO}_3/\text{DMF}$

WHEN R' = OSu
 n = alkyl chain, W = NH₂, X = O, S
 DMF/ROOM TEMP.

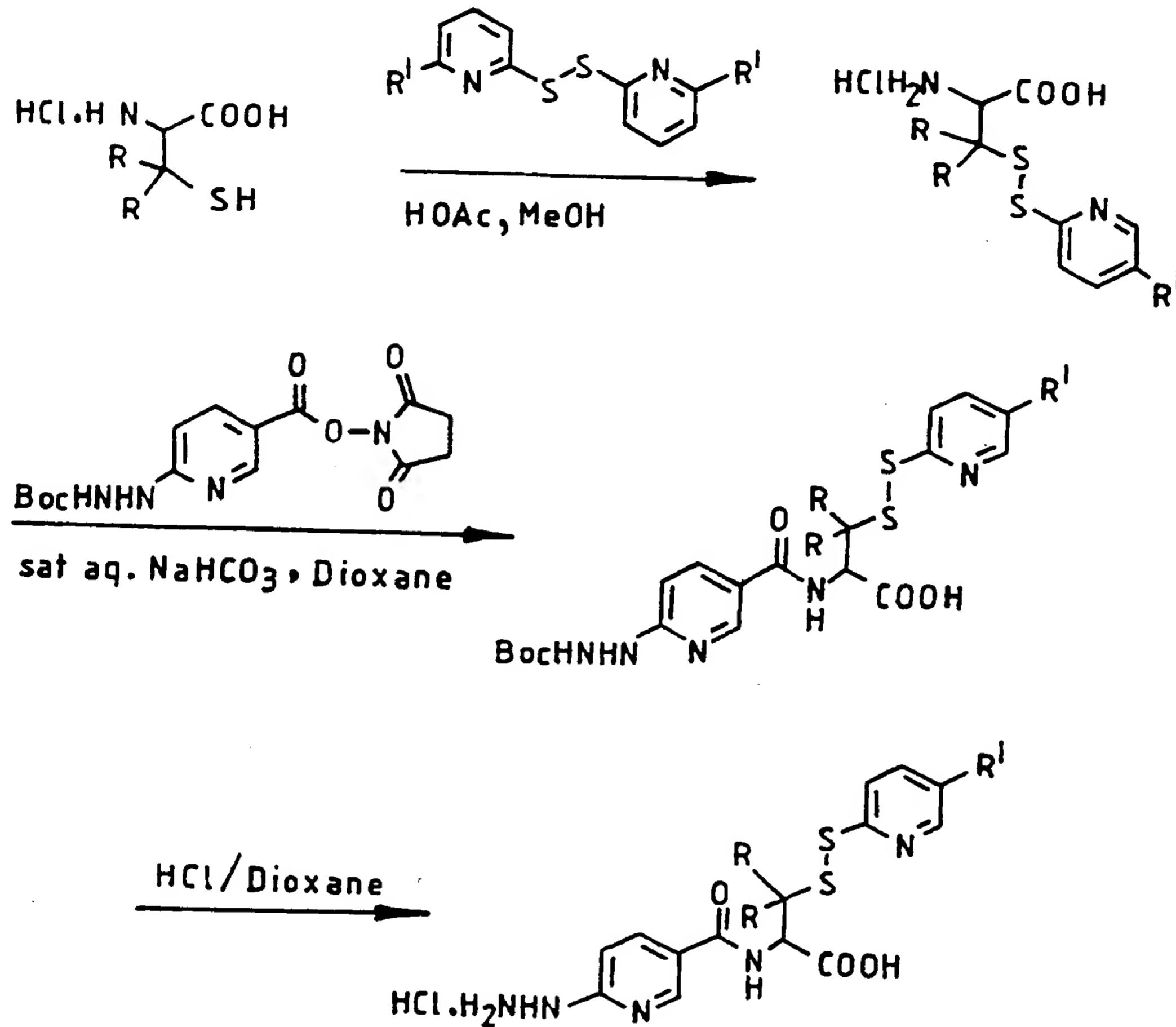


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SCHEME 3

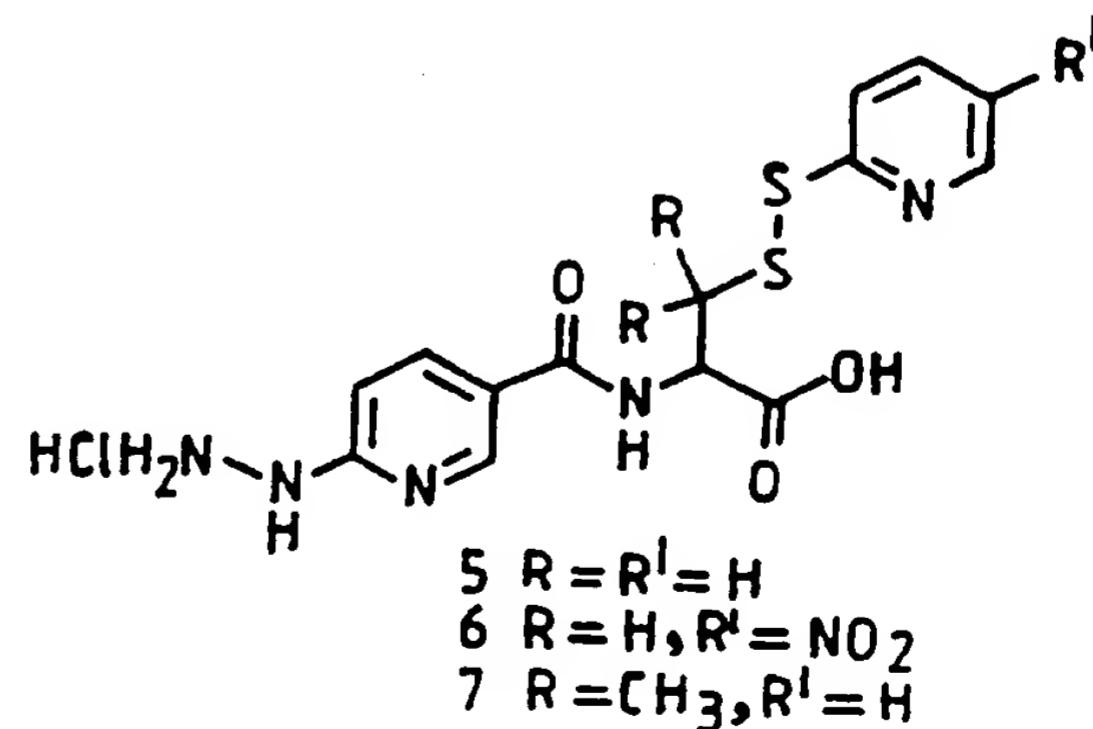
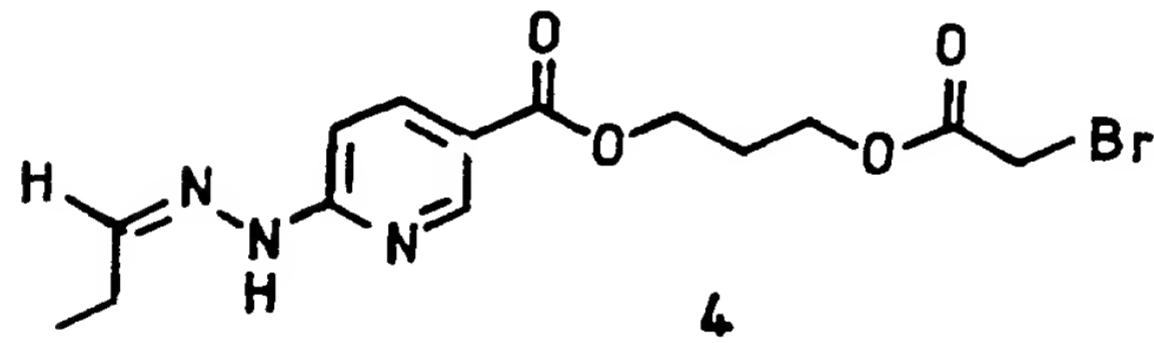
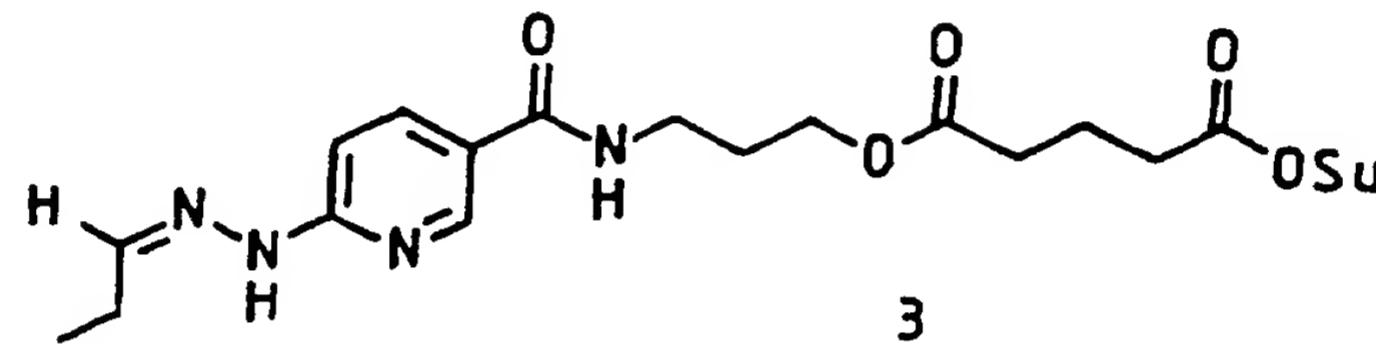
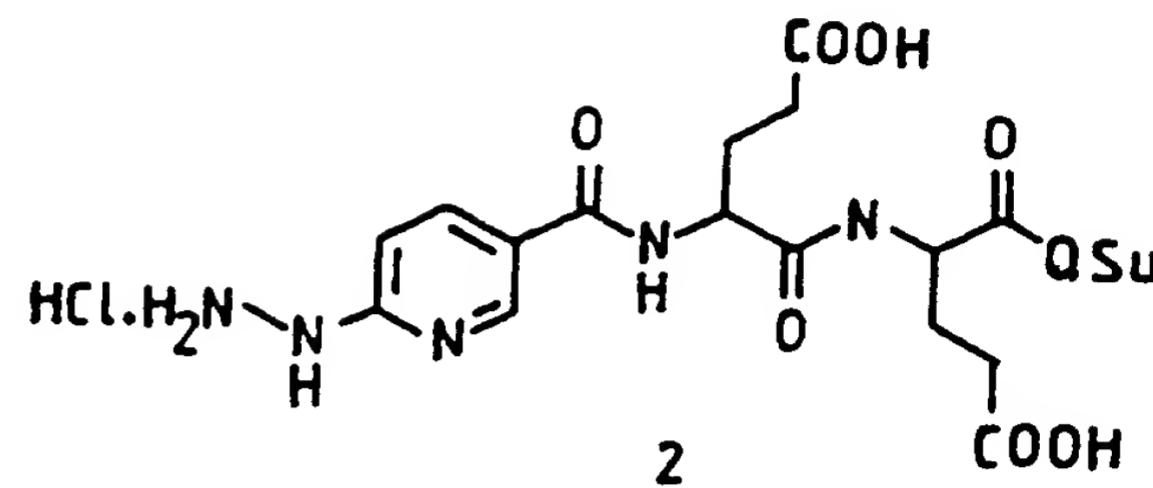
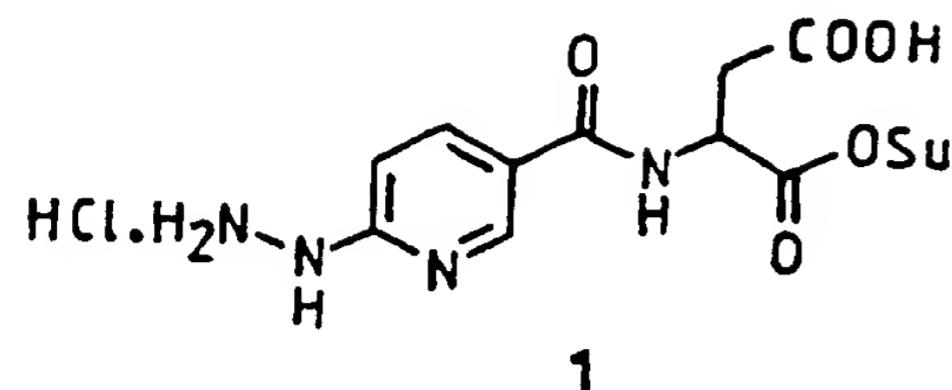
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SCHEME 4

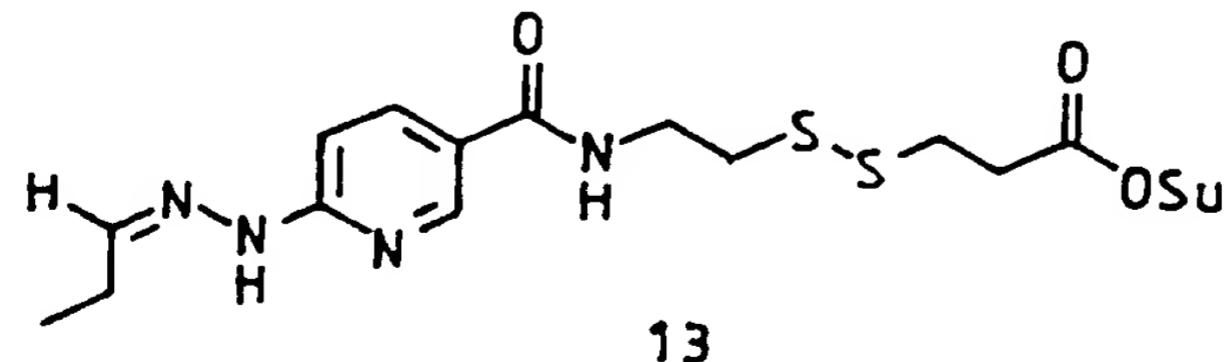
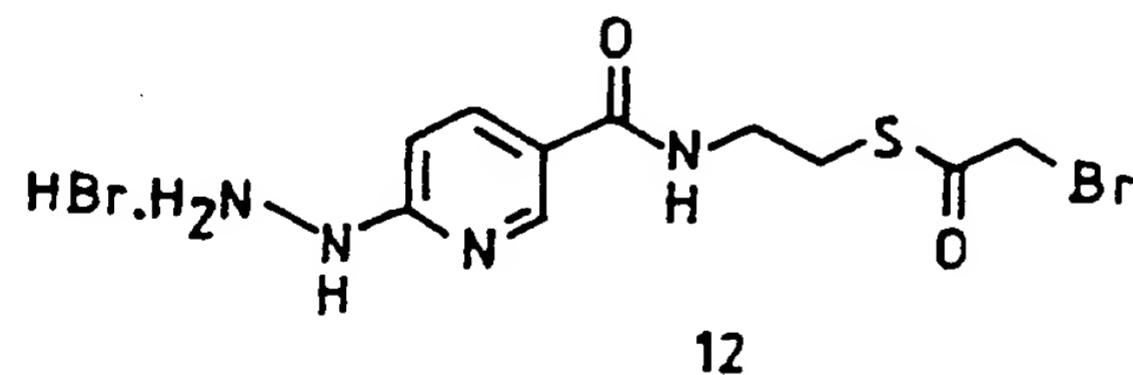
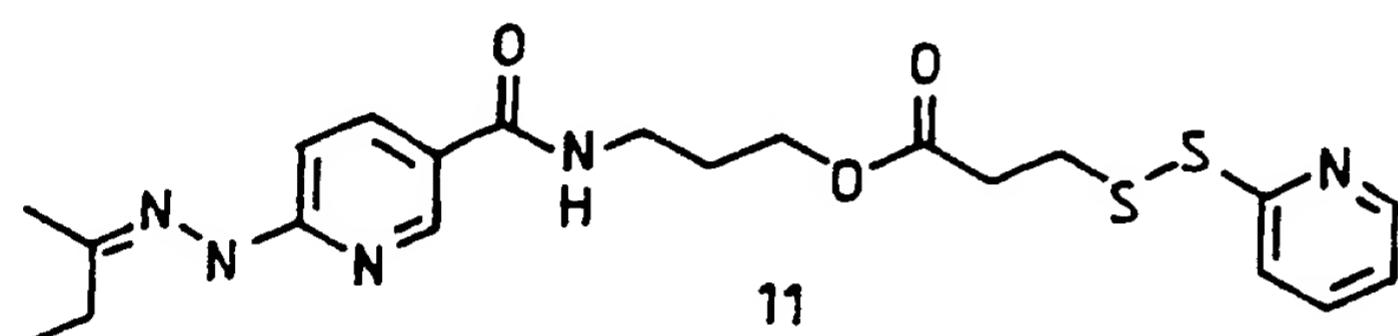
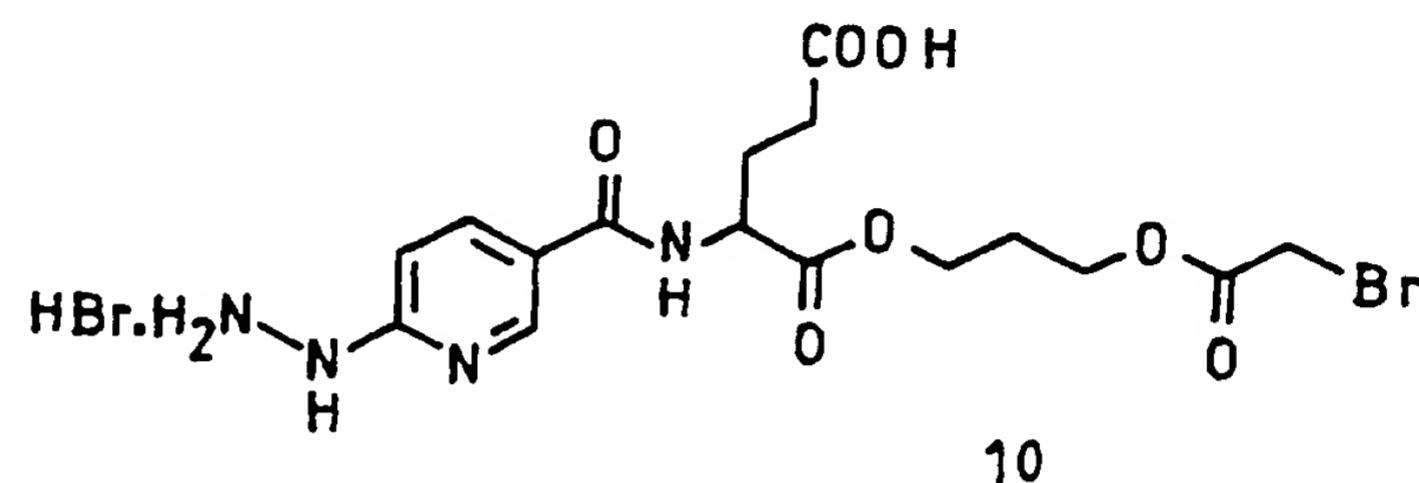
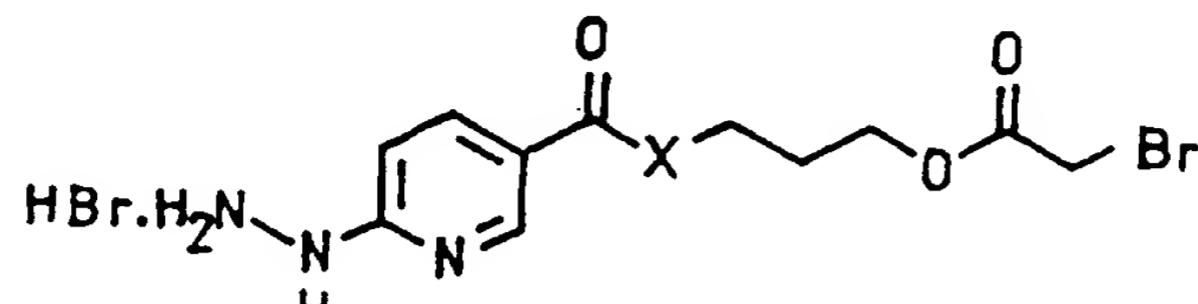


5 $\text{R}=\text{R}'=\text{H}$
 6 $\text{R}=\text{H}, \text{R}'=\text{NO}_2$
 7 $\text{R}=\text{CH}_3, \text{R}'=\text{H}$

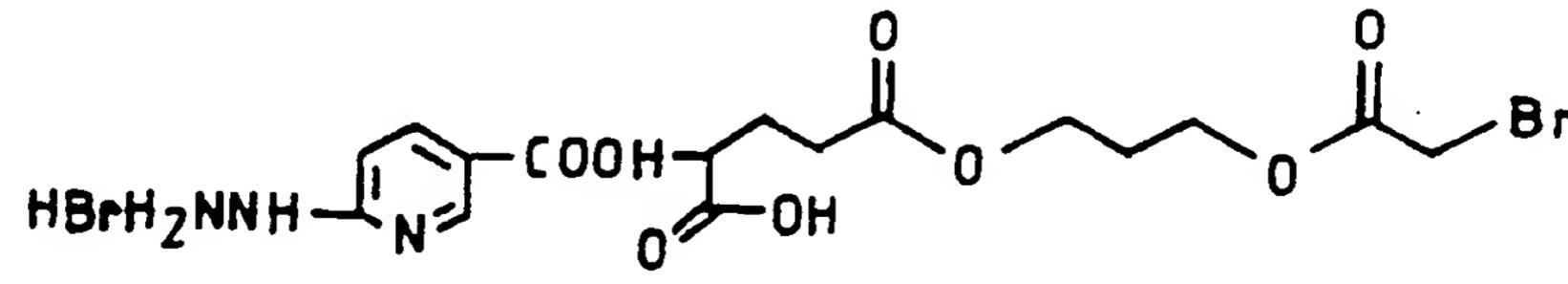
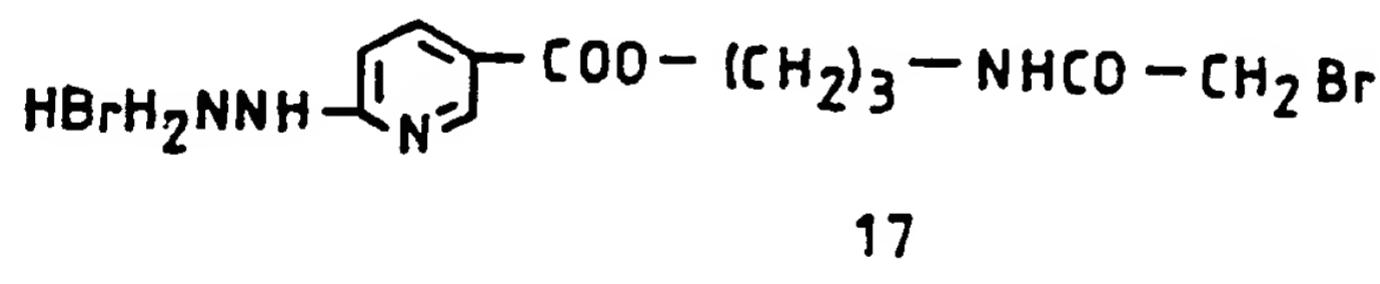
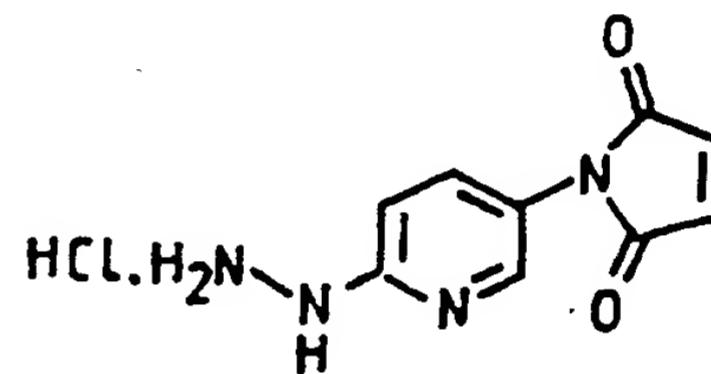
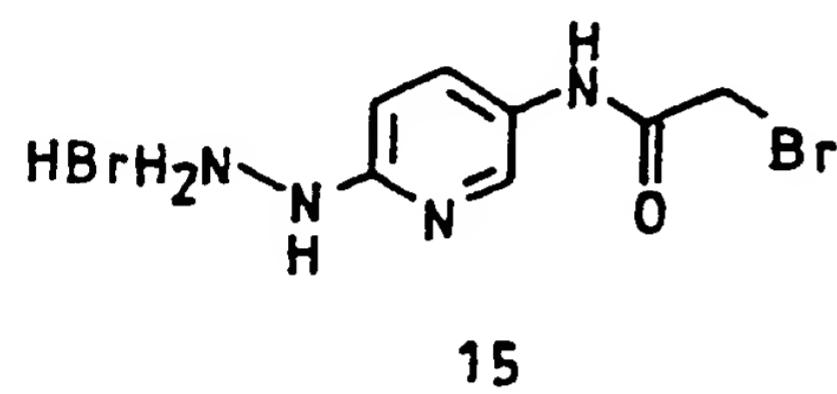
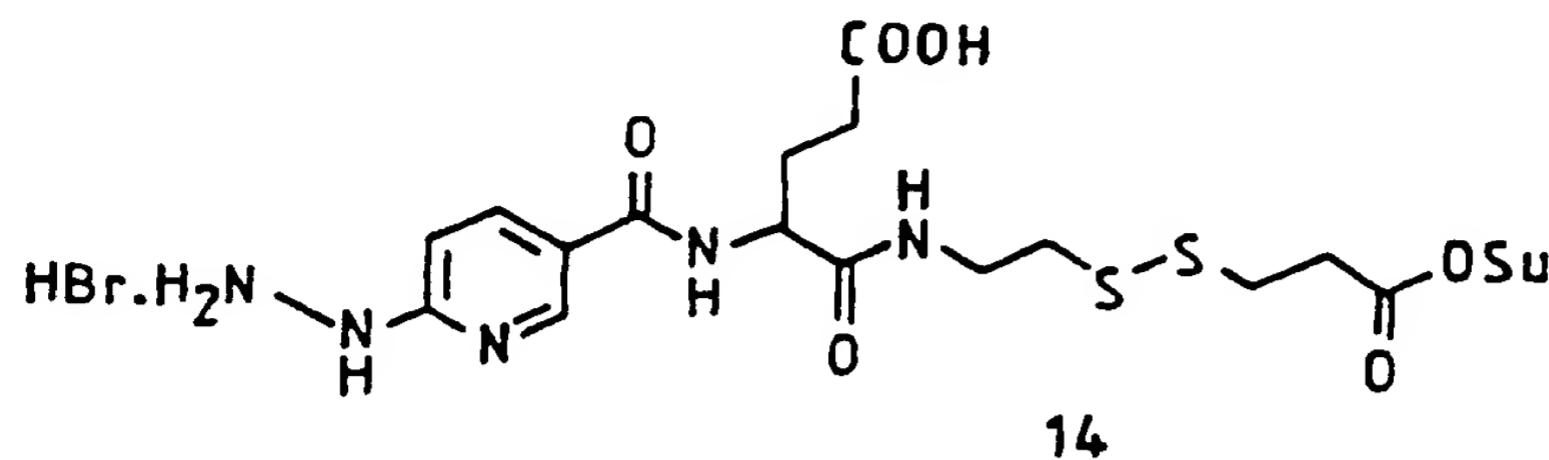
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/02259A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07D213/82 A61K31/44 C07D213/80 C07D213/77 A61K31/465

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 384 769 (JOHNSON MATTHEY PUBLIC LTD. COMPANY) 29 August 1990 cited in the application ---	
A	EP,A,0 247 866 (MALLINCKRODT INC.) 2 December 1987 ---	
A	WO,A,91 09876 (NEORX CORP.) 11 July 1991 & US,A,5 112 953 (NEORX CORP.) cited in the application -----	

 Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentdienst 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

DE JONG, B

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Information on patent family members

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